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MEMORY AND MOTIVATIONAL/ EMOTIONAL PROCESSES

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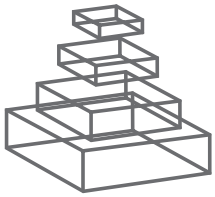

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MEMORY AND MOTIVATIONAL/ EMOTIONAL PROCESSES

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It is well established that memory for emotional information is generally better than for neutral information. This Research Topic comprises a set of papers focusing on memory and its relation with motivational and emotional processes, ranging from electroencephalographic evidences of emotional modulation of memory systems, to the role of neurotransmitters/ neuromodulators (i.e. endocannabinoid, glucocorticoid, serotonin, noradrenergic, dopaminergic systems), and second messengers on emotional memory, and the specific involvement of cerebral areas on the relation between memory and motivational/emotional processes (i.e. prefrontal cortex, amygdala, accumbens). In particular, some of the topics discussed in this special issue will include: cortical activity correlates of emotional modulation of memory systems, interactions between ascending vagal fibers and central noradrenergic systems in modulating memory for emotionally arousing events, involvement of prefrontal /accumbal catecholamine system in processing emotional and motivational salience, role of both negative and positive emotional arousal in increasing persistence of consolidated memories through modulation of second messengers and the involvement of emotional arousal in the activation of amygdala projections, that can then modulate different types of memory.

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EDITORIAL
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Memory and motivational/emotional processes

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As we know from our own experiences and the findings of many studies, emotional events are remembered with greater accuracy, vividness, and persistency compared to events lacking an emotional component (LaBar and Cabeza, 2006; Roozendaal and McGaugh, 2011). How emotional memory is controlled and regulated? This question has fascinated scientists and clinicians for a long time; in fact, the field focused on memory and motivational/emotional processes represents one of the fastest growing areas of neuroscience research. The selectivity that arousal creates is generally beneficial, as emotionally arousing situations in our lives are worth remembering, so that they can be savored and/or instructive. From an evolutionary point of view, it seems logical that a confrontation with an emotionally arousing event, such as a stressful one, is better remembered than a neutral one, resulting in a more adequate motivation to react in a similar situation.

Why emotional arousal enhances memory? Taking into account that neural processes initiated by an experience perseverate and consolidate over time, a possible explanation is that emotional arousal could activate neurobiological processes that modulate the consolidation of memories of recent experiences.

This special issue includes original papers and review articles that cover cutting-edge research in the interplay between memory, motivation, and emotion, providing the reader with what is up and coming with respect to research findings, theoretical advances, and methodological techniques. Many of the current "hot" topics in the field are covered, including the involvement of specific cerebral regions on the interaction between memory and motivational/emotional processes, the contribution of neurotransmitters and neuromodulators, and the role of arousal and stress.

The enhanced memory for emotional events has been attributed to the involvement and interaction of brain regions, in particular between the amygdala and other areas such as the hippocampal formation and prefrontal cortex (Phelps, 2004; Richter-Levin, 2004; McIntyre et al., 2012). The amygdala is active during emotional situations, and this activity influences the encoding and consolidation of the memory trace for the emotional event (McGaugh, 2004). On the light of previous evidence, some papers of this special issue focus on the role of specific neural regions in the interplay between memory and motivational/emotional processes, such as cortical and mesocorticolimbic areas (Martínez-Moreno et al., 2011; El Rawas et al., 2012; Holloway-Erickson et al., 2012; Puglisi-Allegra and Ventura, 2012), hippocampal formation (Hori et al., 2011; Garín-Aguilar et al., 2012), amygdala, substantia nigra, and striatum (Salado-Castillo et al., 2011; Wolf et al., 2011), septal nuclei (Matsuyama et al., 2011), nucleus accumbens (Núñez-Jaramillo et al., 2012), and autonomic nervous system (Garcia et al., 2011). Another group of papers analyzes the role and interaction of neurotransmitters and neuromodulators, such as catecholamines (Puglisi-Allegra and Ventura, 2012), endocannabinoids (Campolongo et al., 2012), acetylcholine and glucocorticoids (Fornari et al., 2012; Sánchez-Resendis et al., 2012) on memory and motivational/emotional processes. In order to highlight the impact of motivation and emotion on memory, functional neuroimaging techniques were used, including multichannel electroencephalography (EEG) (Arnone et al., 2011; Garcia et al., 2011; Uribe et al., 2011) and functional magnetic resonance imaging (fMRI) (Jepma et al., 2012; Rosales-Lagarde et al., 2012). Moreover, taking into account that recent studies have revealed seemingly large, but previously unsuspected, sex-related influences on the well-known mechanism that emotional events are better memorized than neutral events, this special issue includes evidence of sex-related differences in memory and talkativeness for emotional stimuli (Arnone et al., 2011). Finally, considering that in recent years a key conceptual issue, that warrants attention, is the fact that many studies examining emotional memory have focused on the highly arousing nature of emotional stimuli or experimental contexts, as the key component contributing to the enhancement of memory, some papers of this special issue discuss the involvement of arousal and stress in the interplay between memory, motivation, and emotion (Cruciani et al., 2011; Uribe et al., 2011; Packard and Goodman, 2012).

In conclusion, we hope that this special issue have provided evidence of the important and rapid progresses in this very interesting and relevant topic, and may give a significant contribution to the knowledge of how memory can be affected by emotional experiences, and related motivation. Then, taking into account that this emergent field is in continuous and fast growing, we strongly hope that the present special issue may *motivate* many neuroscientists to conduct other studies, paving the way for the next great theories and advances.

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Reward contingency modulates neuronal activity in rat septal nuclei during elemental and configural association tasks

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It has been suggested that septal nuclei are important in the control of behavior during various reward and non-reward situations. In the present study, neuronal activity was recorded from rat septal nuclei during discrimination of conditioned sensory stimuli (CSs) of the medial forebrain bundle associated with or without a reward (sucrose solution or intracranial self-stimulation, ICSS). Rats were trained to lick a spout protruding close to the mouth just after a CS to obtain a reward stimulus. The CSs included both elemental and configural stimuli. In the configural condition, the reward contingency of the stimuli presented together was opposite to that of each elemental stimulus presented alone, although the same sensory stimuli were involved. Of the 72 responsive septal neurons, 18 responded selectively to the CSs predicting reward (CS^+-related), four to the CSs predicting non-reward (CS^0-related), nine to some CSs predicting reward or non-reward, and 15 non-differentially to all CSs. The remaining 26 neurons responded mainly during the ingestion/ICSS phase. A multivariate analysis of the septal neuronal responses to elemental and configural stimuli indicated that septal neurons encoded the CSs based on reward contingency, regardless of the stimulus physical properties and were categorized into three groups; CSs predicting the sucrose solution, CSs predicting a non-reward, and CSs predicting ICSS. The results suggest that septal nuclei are deeply involved in discriminating the reward contingency of environmental stimuli to manifest appropriate behaviors in response to changing stimuli.

Keywords: conditioning, intracranial self-stimulation, motivation, non-reward, reward

INTRODUCTION

Septal nuclei receive afferents from various areas in the limbic system not only from the hippocampal formation, but also from the amygdala, entorhinal, and cingulate cortex, which are all involved in various higher brain functions such as cognition and memory. In turn, the septal nuclei send efferents to the hypothalamus and brainstem, which play an important role in emotional expression or behavioral manifestations such as emotional behavior, hormone release, and autonomic reactions (see review by Risold and Swanson, 1997b). Thus, the septal region acts as an interface between these higher cognitive and lower executive systems (Swanson et al., 1987).

Septal nuclei have a functionally strong relations to the hippocampal formation and hypothalamus. Septal or fornical lesions impair spatial learning and memory (Thomas and Gash, 1986; Numan and Quaranta Jr., 1990; Gaffan et al., 1991), which is comparable to the effects of hippocampal lesions (Gray, 1987). Furthermore, septal lesions alter food and water intake, which is also observed after hypothalamic lesions (Harvey and Hunt, 1965; Lorens and Kondo, 1969; Stoller, 1972). Electrically stimulating septal nuclei results in lowered arousal, hypoactivity, reduced rates of response, and even sleep and induces autonomic responses such as decreased blood pressure, cardiac deceleration, and inhibition of pituitary–adrenal activity as well as somatomotor effects in rats and cats (Malmo, 1961, 1965; Covian et al., 1964; Holdstock, 1967; Baldino et al., 1988). Furthermore, abnormalities in the septal region have been associated with schizophrenia and depression (Zeman and King, 1958; Averback, 1981; Brisch et al., 2011). Evidence suggests that septal nuclei control emotional behavior and autonomic and hormonal functions by modulating hypothalamic and lower brainstem activity, based on higher cognitive and mnemonic information received through reciprocally dense connections with other areas of the limbic system including the hippocampal formation.

Furthermore, the septal region was the first area where intracranial self-stimulation (ICSS) behavior was observed in rats (Olds and Milner, 1954). Septal nuclei have intimate connections to brain regions involved in goal-directed behaviors for various reward, such as the nucleus accumbens, medial prefrontal cortex, and ventral tegmental area (VTA) and are implicated in amphetamine, morphine, phencyclidine, and lysergic acid diethylamide abuse (Sheehan et al., 2004). These craving behaviors may be mediated partly through an interaction between the septal nuclei and the dopaminergic system (Merrer et al., 2007). Additionally, nearly all antipsychotic antidepressant drugs affect septal neuron activity (Sheehan et al., 2004). Taken together, these results suggest that septal neuron activity is an important determinant of behavioral manifestations during various reinforcing and non-reinforcing situations.

In the present study, neuronal activity was recorded from rat septal nuclei while rats performed elemental and configural association tasks to elucidate the information processing mechanisms in the septal nuclei that control motivated and emotional behaviors.

During these tasks, conditioned sensory stimuli (CSs) included both elemental (auditory or visual) and configural (simultaneously presented auditory and visual) stimuli. In one case, each stimulus predicted reward when presented alone but predicted non-reward when presented together. In the other case, each stimulus predicted non-reward when presented alone but predicted reward when presented together. Previous anatomical and behavioral studies suggest that septal nuclei may integrate information from other brain regions to influence behavioral output, particularly during reinforcing situations. If the septal neurons are involved in behavioral output during reinforcing situations, they would respond to the CSs in terms of reward availability regardless of the physical properties of the CSs.

MATERIALS AND METHODS

SUBJECTS

Twenty-six male albino Wistar rats, weighing 270–330 g (10–16 weeks old; SLC, Hamamatsu, Japan), were used. The housing area was temperature controlled at 23°C and maintained on a 12-h light–dark cycle. Prior to surgery, rats were individually housed in clean cages with free access to water and laboratory chow. All efforts were made to minimize the number of animals used and their suffering.

SURGERY

Surgery was performed under aseptic conditions in two stages. First, a cranioplastic cap was attached to the skull. After a recovery and training period, a permanent indifferent electrode was implanted. The rats were treated in strict compliance with the policies of the National Institutes of Health on the Care of Humans and Laboratory Animals and the Guidelines for the Care and Use of Laboratory Animals at the University of Toyama.

As described in our previous studies (Uwano et al., 1995; Nishijo et al., 1998), the head restraint system of Nishijo and Norgren (1990, 1991, 1997), modified from a method described by Ono et al. (1985), was used. After being anesthetized (sodium pentobarbital, 40 mg/kg, i.p.), the rats were mounted in a stereotaxic apparatus with the skull leveled between the bregma and lambda suture points. The cranium was exposed, and 2–3 mm of the temporal end of the bilateral temporal muscle was removed, and seven small, sterile, stainless screws were threaded into holes in the skull to serve as anchors for cranioplastic acrylic. Stainless-steel wires were soldered onto two screws as a ground. Two bipolar electrodes were implanted into the lateral hypothalamic area (*A*, –4.3 from bregma; *L*, ± 1.2; *V*, 8.4) to intracranially stimulate the medial forebrain bundle, according to the atlas of Paxinos and Watson (1986). After covering the cut end of the temporal muscle with overlying skin, the cranioplastic acrylic was built up on the skull and molded around the conical ends of two sets of stainless-steel bars that had a single steel bar on one end and two bars on the other end. Once the cement had hardened, these bars were removed, leaving a negative impression of the double end on each side of the acrylic block. During subsequent surgery or during a recording session, the double end of these artificial earbars was pressed into the indentations in the acrylic block, while the single end was inserted into the normal earbar slots in the stereotaxic instrument and rigidly attached (**Figure 1A**). Hence, these artificial earbars served the same function as regular earbars but could be used in unanesthetized animals because they did not involve a painful insertion into the ear canal. A short length of 27-gage stainless-steel tubing was embedded into the cranioplastic acrylic near the bregma to serve as a reference pin during chronic recording. After surgery, an antibiotic (gentamicin sulfate, Gentacin® injection, Schering-Plough, Osaka, Japan) was administered topically and systematically (2 mg, i.m.).

After recovery from surgery (10–14 days) and training (2 weeks; see below), rats were anesthetized (sodium pentobarbital, 40 mg/kg, i.p.) again and mounted with the artificial earbars. A hole (diameter, 2.8–3.0 mm) was drilled through the cranioplastic and the underlying skull (*A*, –1.5 to 1.5 from bregma; *L*, 0.2 to 3.0 right) for chronic recording. The exposed dura was excised, and the hole was covered with hydrocortisone ointment (Rinderon-VG® ointment, Shionogi Co., Ltd., Tokyo, Japan), or one or two drops of chloramphenicol (Chloromycetin® succinate, Sankyo Co., Ltd., Tokyo, Japan) solution (0.1 g/ml) were dropped into the hole. The hole was covered with a sterile Teflon sheet and sealed with epoxy glue. A second small hole (diameter, 1.5 mm) was then drilled just contralateral to the recording hole. A stainless-steel wire (diameter, 130 μm), which was insulated except at the cross section of the tip, was implanted near the lateral end of the left lateral septal nuclei through the hole to serve as an indifferent electrode. This hole was then filled with cranioplastic acrylic. After the animal recovered (5–7 days), it was placed back on the water-deprivation regimen (see Training and Task paradigms).

TRAINING AND TASK PARADIGMS

Before surgery, the rats were acclimated by handling and became accustomed to being placed into a small, plastic restraining cage for brief periods. The threshold level for ICSS was determined after recovery from the first stage of surgery (Ono et al., 1985), and three rats for which the threshold exceeded 300 μA were excluded. The remaining rats were reacclimated to the plastic enclosure and placed on a 22-h water-deprivation regimen. While in the enclosure (1–2 h daily), they had access to a spout from which they learned to take fluid within 1–2 days, which was initially 0.3 M sucrose. Subsequently, their heads were rigidly and painlessly fixed by inserting the artificial earbars into the impressions in the cranioplastic cap. While restrained, the rats were trained to lick a spout, which was automatically extended close to their mouths for 2 s to obtain the sucrose solution or ICSS. The rats were then trained to discriminate between conditioned elemental (auditory or visual) or configural stimuli to obtain a reward. Sensory stimuli included auditory (1200, 2800, or 4300 Hz), visual (white light), and configural (simultaneous presentation of tone and light) stimuli (**Table 1**). A mid range speaker, located 1 m above the rat, delivered the auditory stimuli, and two white lights, 3 cm in front of each eye, delivered the visual stimuli. Licking was signaled by a photoelectric sensor triggered by the tongue. The rats were trained to lick the spout to obtain a reward (sucrose solution or ICSS; reward task; **Figure 1B**). A 1200-Hz tone (Tone 1), a white light in front of the right eye (Light 1), or the simultaneous presentation of a 2800-Hz tone (Tone 2) and a white light in front of the left eye (Light 2; Tone 2 + Light 2: configural stimulus) signaled availability of the 0.3-M sucrose solution. A 4300-Hz tone (Tone 3) signaled the ICSS (0.5 s train of 100 Hz, 0.3 ms capacitor-coupled negative square wave

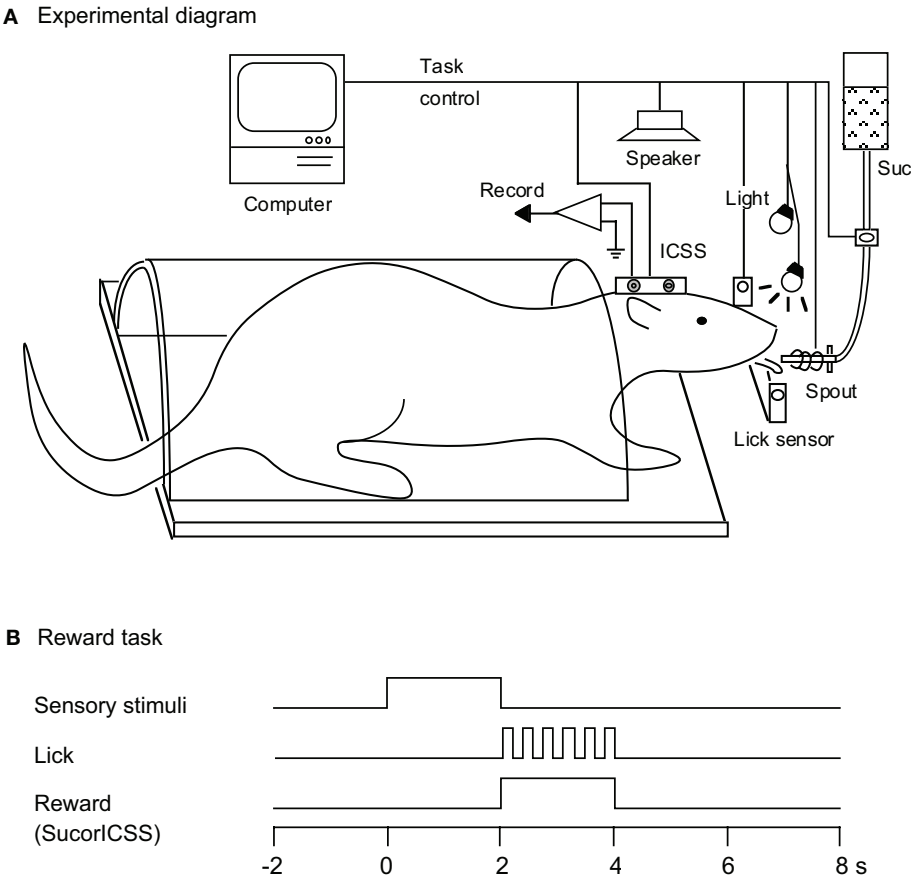

FIGURE 1 | Experimental diagram. (A) Rats were prepared for chronic recording by forming dental cement receptacles to accept fake earbars. Electrodes were implanted in the lateral hypothalamic area for intracranial self-stimulation (ICSS) of the medial forebrain bundle. Rats were trained to lick when the spout was automatically placed close to their mouth. Licking was signaled by a photoelectric sensor triggered by the tongue. Auditory and visual CSs were presented by a speaker above the rat's head and by a light in front of each eye, respectively. A sucrose solution was delivered from a spout, and electric current for the ICSS was applied to the stimulating electrode. Suc, 0.3 M sucrose solution. **(B)** Time chart for the reward task. During this task, the conditioned sensory stimulus (tone, light, or tone + light: configural) associated with or without a reward stimulus (sucrose solution or ICSS) was presented for 2 s prior to placing the spout close to the rat's mouth.

Table 1 | Summary of various conditioned sensory stimuli associated with or without reward.

	Conditioned stimulus	Reward
Auditory stimuli	Tone 1 (1200 Hz)	Sucrose solution
	Tone 2 (2800 Hz)	No reward
Visual stimuli	Tone 3 (4300 Hz)	ICSS
	Light 1 (right)	Sucrose solution
Configural stimuli	Light 2 (left)	No reward
	Tone 1 + Light 1	No reward
	Tone 2 + Light 2	Sucrose solution

Tones 1–3 correspond to pure tones of 1200, 2800, 4300 Hz, and Lights 1–2 to visual stimulation by white lights located in front of right and left eyes, respectively.

pulses). Tone 2, Light 2, or simultaneous presentation of Tone 1 and Light 1 (Tone 1 + Light 1: configural stimulus) signaled that no reward was available.

Training with or without a reward was conducted in one block of 10 or 20 trials. The total number of trials per day was 400–500 in 4 h from 16:00 to 20:00. Throughout the training and recording period, a rat was permitted to ingest 20–30 ml of sucrose solution while in the restrainer. If the rat failed to consume 30 ml of the sucrose solution while restrained, it was given the remainder when it was returned to its home cage. Only sucrose solution was available during the task.

Our previous study using the same paradigm in rats reported that the latencies of licking after offset of the CS became shorter than 300 ms after learning the association between the CS and reward (Toyomitsu et al., 2002). After the rats had learned the tasks described above (i.e., when licking latencies became shorter than 300 ms), septal neurons were recorded from during performance of these elemental and configural association tasks.

ELECTROPHYSIOLOGICAL RECORDING

An individual rat was usually tested every other day. After being placed in the enclosure, the ointment was removed, and a glass-insulated tungsten microelectrode (Z = 1.0–1.5 MΩ at 1000 Hz)

was stereotaxically inserted stepwise with a pulse motor-driven manipulator (SM-20, Narishige, Tokyo, Japan) into various parts of the right septal region. Extracellular neuronal and electromyographic (EMG) activity was passed through a dual channel differential amplifier with a preamplifier (DPA-220, DIA Medical System Co., Tokyo, Japan), monitored on an oscilloscope, and recorded on a data recorder (RT-145T Dat Data Recorder, TEAC, Tokyo, Japan). Neuronal activity was quantified with a two-level voltage discriminator. The analog signal, the trigger levels, and the output of the discriminator were monitored continuously on an oscilloscope during analysis. The discriminator output pulses were accumulated and displayed as peri-stimulus histograms by an on-line minicomputer (ATAC-450, Nihon Kohden, Tokyo, Japan). Another computer (PC-98 21 Bp, NEC, Tokyo, Japan) stored the events and times of the trigger signals, output pulses from the discriminator, and lick signals for display of rasters and histograms off-line.

DATA ANALYSIS

Both neuronal and behavioral data of each trial were counted from the peri-stimulus histograms in successive 100-ms bins for three phases: a pre-trial control phase (2 s), a CS phase (2 s), and a reward (or non-reward) phase (2 s). Neuronal activities were compared among firing rates during these three phases. Neuronal excitation or inhibition was determined by the *post hoc* test (new multiple range test, $p < 0.05$) between the mean firing rate during the control phases (mean spontaneous firing rate) and that during each CS or reward phase after a one-way analysis of variance (ANOVA). The new multiple range test was selected because of its protection from Type II errors (Duncan, 1955). Each neuronal response to each CS was also compared with a one-way ANOVA and the *post hoc* test.

The mean firing rates among various neuronal types and recording sites were also compared by one-way ANOVA and *post hoc* tests ($p < 0.05$).

Neuronal data were also treated with multidimensional scaling (MDS) to examine the relationships among seven CSs represented by the septal neurons. MDS is a method to simplify the relationships within a complex array of data; it constructs a geometric representation of the data to show the relationship between stimuli represented by the data matrix (see Young, 1987 for more details). MDS employs the metric ratio and Euclidean model (SYSTAT statistical package, Guttman scaling method). The similarities (Pearson's correlation coefficients) between each possible pair of CSs were calculated. The MDS program computed the Pearson's correlation coefficients between all possible pairs of two CSs to give interstimulus relationships by plotting the relative positions of seven CSs in two-dimensional space. The statistical criteria, categories, and numerical analyses were identical to those used in two previous studies that used correlation coefficients for the MDS (Nishijo and Norgren, 1990, 1991).

HISTOLOGY

After the last recording session, rats were anesthetized again with sodium pentobarbital (50 mg/kg, i.p.) and several small electrolytic lesions (20 μA for 20 s) were made stereotaxically around the recorded sites with a glass-insulated tungsten microelectrode. Rats were then given an overdose of anesthetic and perfused transcardially with heparinized 0.9% saline followed by 10% buffered formalin. The brain was removed, and cut into 50 μm frontal sections with a freezing microtome. Sections were stained with cresyl violet. All marking and stimulation sites were then carefully verified microscopically. Positions of neurons were stereotaxically located on the actual tissue sections and plotted on the corresponding sections of the atlas of Paxinos and Watson (1986).

The rat septal region consists of medial (MS), lateral (LS), and posterior divisions (PS) based on topography, cytoarchitecture, and connections (Jakab and Leranth, 1995). The MS included the medial septal nucleus and diagonal band of Broca. The LS consisted of dorsal, intermediate, and ventral parts. The PS included the septofimbrial nucleus and triangular septal nucleus. The classification and terminology of these septal subnuclei were based on the atlas of Paxinos and Watson (1986).

RESULTS

CLASSIFICATION OF THE NEURONS IN THE SEPTAL NUCLEI

Recording was performed over a period of 1–2 months for each rat. The activities of 307 neurons in and around the septal nuclei were recorded during the reward task. Of these, 284 neurons were located in septal nuclei. **Table 2** summarizes the response patterns of these 284 neurons resulting from statistical analyses by one-way ANOVA and *post hoc* comparisons. Seventy-two neurons (25.4%) responded during one or more phases of the task: 42 with excitation and 30 with inhibition. These 72 responsive neurons were classified into two types based on response magnitudes during the CS and reward phases; CS-related and ingestion/ICSS-related. When the neurons responded during both the CS and reward phases, ingestion/ICSS-related neurons were defined as neurons with responses during the reward phase that were more than twice as large as the responses during the CS phase. There were 46 CS-related and 26 ingestion/ICSS-related neurons. The 46 conditioned stimulus-related neurons were further subclassified based on their responsiveness to each CS. Eighteen CS-related neurons responded selectively to the CSs predicting reward (CS^+-related), four to the CSs predicting non-reward (CS^0-related), and nine differentially to some CSs predicting

Table 2 | Classification of the neurons recorded in the septal nuclei.

Classification	Number of neurons (E/I)			
	LS	MS	PS	Total
Conditioned stimulus-related	41 (25/16)	2 (0/2)	3 (2/1)	46 (27/19)
Differential	26 (18/8)	2 (0/2)	3 (2/1)	31 (20/11)
CS^+-related	15 (11/4)	2 (0/2)	1 (1/0)	18 (12/6)
CS^0-related	4 (3/1)	0	0	4 (3/1)
Miscellaneous	7 (4/3)	0	2 (1/1)	9 (5/4)
Non-differential	15 (7/8)	0	0	15 (7/8)
Ingestion/ICSS-related	23 (14/9)	3 (1/2)	0	26 (15/11)
Total responded	64 (39/25)	5 (1/4)	3 (2/1)	72 (42/30)
No response	185	19	8	212
Total	249	24	11	284

E, excitation; I, inhibition; LS, lateral septal nucleus; MS, medial septal nucleus; PS, posterior septal nucleus. Numbers in parentheses indicate numbers of neurons.

reward or non-reward (miscellaneous). The remaining 15 neurons responded non-differentially to all CSs (non-differential) regardless of reward contingency.

CS$^+$-RELATED NEURONS

Eighteen CS$^+$-related neurons responded selectively during presentation of the CSs predicting reward (**Table 2**): the activity of 12 and 6 neurons increased and decreased, respectively, during the CS phase. A typical example of this type of neuron is shown in **Figure 2**. Raster displays and each dot below a raster line indicate neuronal activity and one lick. Upper and lower histograms show accumulated neuronal activity and licks (**Figures 2A–G**). This neuron displayed excitatory responses to Tone 1 (**Figure 2A**), Light 1 (**Figure 2B**), Tone 3 (**Figure 2D**), and Tone 2 + Light 2 (**Figure 2G**), predicting sucrose solution or ICSS, but not to Tone 1 + Light 1 (**Figure 2C**), Tone 2 (**Figure 2E**), or Light 2 (**Figure 2F**) predicting non-reward. The mean firing rates during presentation of the CSs predicting reward (Tone 1, Light 1, Tone 2 + Light 2, or Tone 3) were significantly larger than those during the control phase ($p < 0.05$). The mean response magnitudes of the neuron to various CSs are indicated in **Figure 2H**. Statistical comparison using a one-way ANOVA indicated a significant difference in the response magnitudes among the CSs [$F(6, 21) = 20.534$, $p < 0.01$]. *Post hoc* comparisons indicated that the mean firing rates during presentation of the CSs predicting reward (Tone 1, Light 1, Tone 2 + Light 2, or Tone 3) were significantly larger than those during presentation of the CSs predicting non-reward (Tone 1 + Light 1, Tone 2, or Light 2; $p < 0.05$). Thus, the neuron responded only to the CSs associated with reward, but not to the CSs associated with non-reward.

Among the 18 CS$^+$-related neurons, 12 showed similar responsiveness to that of the neuron shown in **Figure 2**. Of the remaining six neurons, one responded to the CSs including the auditory stimulus predicting reward (Tone 1, Tone 3, Tone 2 + Light 2), two to the CSs predicting the sucrose solution (Tone 1, Light 1, Tone 2 + Light 2), and three to the CS predicting ICSS (Tone 3).

CS^0-RELATED NEURONS

Four CS^0-related neurons responded selectively during presentation of CSs, including the visual stimulus predicting non-reward (**Table 2**): the activity of three neurons increased and one decreased during the CS phase. A typical example of this type of neuron is shown in **Figure 3**. The activity of this neuron increased in response to Tone 1 + Light 1 (**Figure 3C**) and Tone 2 (**Figure 3E**), predicting non-reward, but not to Tone 1 (**Figure 3A**), Light 1 (**Figure 3B**), Tone 3 (**Figure 3D**), or Tone 2 + Light 2 (**Figure 3G**), predicting reward, nor to the visual stimulus (Light 2) predicting non-reward. This pattern of responsiveness indicated that the neuron responded to the CSs if the CSs predicted non-reward and included auditory stimuli. The mean firing rates during presentation of the CSs, including the auditory stimuli predicting non-reward (Tone 1 + Light 1 or Tone 2), were significantly larger than those during the control phase ($p < 0.05$). The mean response magnitudes of the neuron during the CS phase are presented in **Figure 3H**. A statistical comparison using a one-way ANOVA indicated a significant difference in the response magnitudes among the CSs [$F(6, 21) = 5.770$, $p < 0.01$]. *Post hoc* comparisons indicated that the mean firing rates during presentation of the CSs predicting non-reward (Tone 1 + Light 1 or Tone 2) were significantly larger than those during presentation of the CSs predicting reward (Tone 1, Light 1, or Tone 2 + Light 2, Tone 3; $p < 0.05$).

Among four CS^0-related neurons, three showed the same responsiveness as that of the neuron shown in **Figure 3**. The remaining neuron responded to all of the CSs predicting non-reward (Tone 2, Light 2, or Tone 1 + Light 1).

MISCELLANEOUS NEURONS

Of 31 differential CS-related neurons, nine showed different responses from those of CS$^+$- and CS^0-related neurons (**Table 2**). Among these nine neurons, one responded to the CSs if they included auditory stimuli regardless of reward contingency (Tone 1, Tone 2, Tone 3, Tone 1 + Light 1, or Tone 2 + Light 2), one only to the configural stimuli (Tone 1 + Light 1, Tone 2 + Light 2), and one to all CSs but it showed significantly different responses to the CSs ($p < 0.05$). The remaining six neurons responded to various CSs, but their responsiveness was unable to be classified.

Figure 4 shows the activity of a neuron that displayed excitatory responses to the CSs if the CS included auditory stimuli. Neuronal activity increased during presentation of Tone 1 (**Figure 4A**), Tone 1 + Light 1 (**Figure 4C**), Tone 3 (**Figure 4D**), Tone 2 (**Figure 4E**), and Tone 2 + Light 2 (**Figure 4G**) but not during presentation of Light 1 (**Figure 4B**) or Light 2 (**Figure 4F**). The mean firing rates during presentation of the CSs, including the auditory stimuli (Tone 1, Tone 1 + Light 1, Tone 2, Tone 2 + Light 2, or Tone 3), were significantly larger than those during the control phase ($p < 0.05$). The mean response magnitudes of the neuron during the CS phase are presented in **Figure 4H**. Statistical comparison by one-way ANOVA indicated a significant difference in the response magnitudes among the CSs [$F(6, 21) = 10.007$, $p < 0.01$].

NON-DIFFERENTIAL CS-RELATED NEURONS

Fifteen neurons responded non-differentially to all CSs with or without reward (**Table 2**): the activity of seven and eight neurons increased and decreased, respectively. during the CS phase. A typical example of a non-differential neuron is shown in **Figure 5**. The activity of this neuron increased in response to all CSs regardless of reward contingency, and the responses continued after the CS phase (**Figures 5A–G**). The mean firing rates during the CSs were significantly larger than those during the control phase ($p < 0.05$). The mean response magnitudes of the neuron during the CS phase are indicated in **Figure 5H**. However, no significant differences among these responses during the CS phase were observed [$F(6, 21) = 1.500$, $p > 0.05$].

MDS ANALYSIS

The responses of 31 differential CS-related neurons to each CS were analyzed by MDS using Pearson's correlation coefficients between all possible CS pairs to elucidate the inter-stimulus relationship among the seven CSs. In the MDS analysis, if response patterns of all differential septal neurons to a given pair of CSs are similar (i.e., Pearson's correlation coefficient between the CSs is nearly 1.0), those CSs are located close to one another in two-dimensional space. In **Figure 6**, the seven CSs were classified into the following three groups in a two-dimensional space based on the distance between the CSs: (i) CSs predicting the sucrose solution (Tone 1,

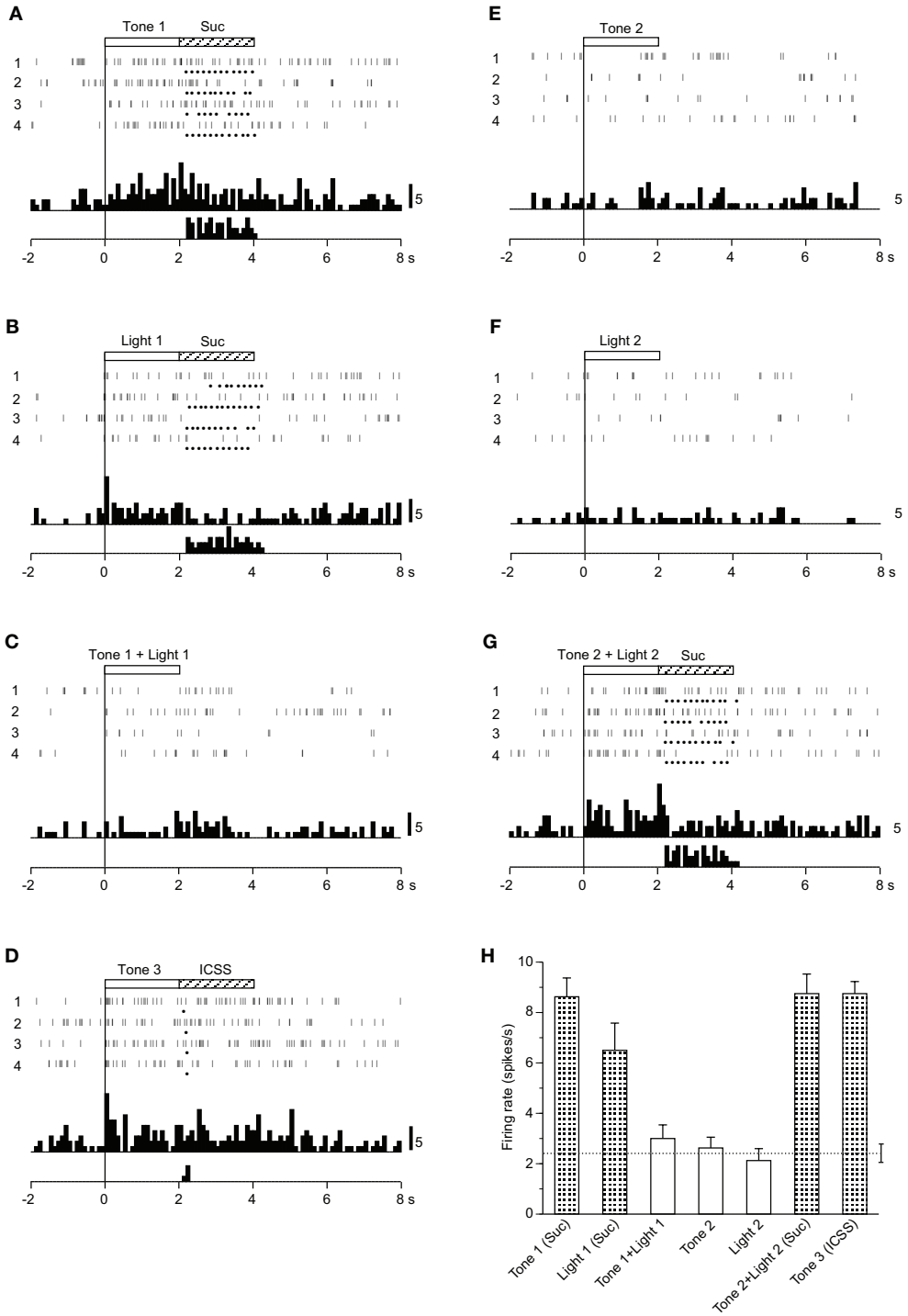

FIGURE 2 | Activity of a conditioned stimulus predicting reward (CS)$^+$-related neuron that responded differently to the conditioned sensory stimuli predicting reward. (A–G) Raster displays and histograms of neuronal responses to Tone 1 predicting the sucrose solution **(A)**, Light 1 predicting the sucrose solution **(B)**, Tone 1 + Light 1 predicting non-reward **(C)**, Tone 3 predicting intracranial self-stimulation (ICSS) **(D)**, Tone 2 predicting non-reward **(E)**, Light 2 predicting non-reward **(F)**, and Tone 2 + Light 2 predicting the sucrose solution **(G)**. Note that neuronal activity increased during presentation of the CS$^+$. White and hatched rectangles at the top indicate CS duration and time of reward, respectively. Each dot below the raster line indicates one lick; each upper histogram shows accumulated neuronal responses; and each lower histogram shows accumulated licks. Time scale, seconds; onset of conditioned stimulus at time 0; minus is a pre-trial control. Each histogram bin, 100 ms. Suc, 0.3 M sucrose solution. **(H)** Histogram of neuronal responses during presentation of each CS (mean firing rate ± SEM). A broken line with error bars indicates the mean spontaneous firing rate and the SEM during the pre-trial control phase. Shaded column, significant difference between the activity during presentation of a given CS and the spontaneous firing rate (new multiple range test after one-way ANOVA, $p < 0.05$).

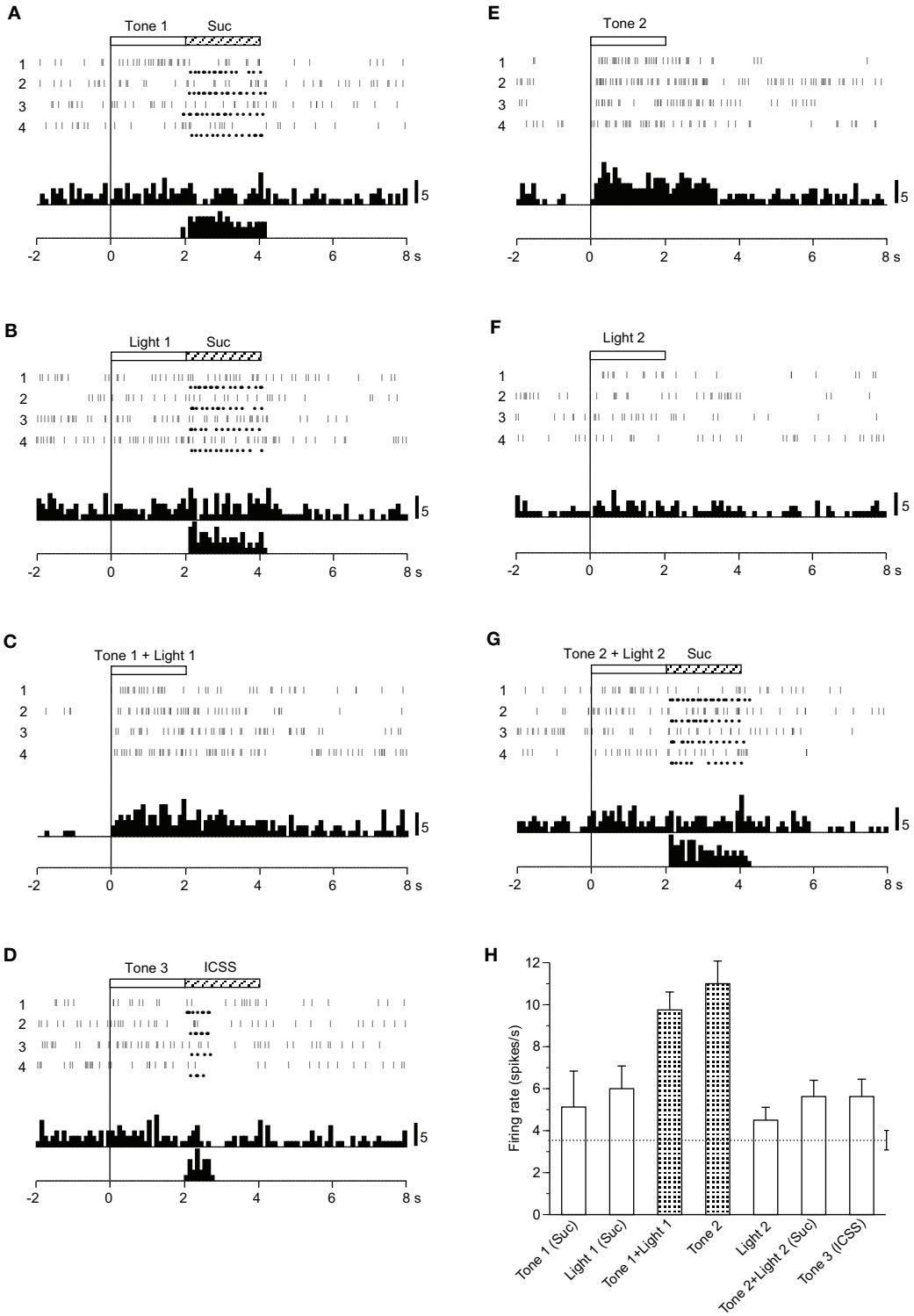

FIGURE 3 | Activity of a conditioned stimulus predicting non-reward (CS^0)-related neuron that responded differently to the conditioned sensory stimuli predicting non-reward. (A–G) Raster displays and histograms of neuronal responses to Tone 1 predicting sucrose solution **(A)**, Light 1 predicting sucrose solution **(B)**, Tone 1 + Light 1 predicting non-reward **(C)**, Tone 3 predicting intracranial self-stimulation (ICSS) **(D)**, Tone 2 predicting non-reward **(E)**, Light 2 predicting non-reward **(F)**, and Tone 2 + Light 2 predicting the sucrose solution **(G)**. Note that neuronal activity increased during presentation of a CS^0 that included the auditory stimulus (i.e., Tone 2 and Tone 1 + Light 1) but not during presentation of a visual CS^0 (Light 2) nor the CS^+ (Tone 1, Light 1, Tone 3, and Tone 2 + Light 2). **(H)** Histogram of neuronal responses during presentation of each CS (mean firing rate ± SEM). Other descriptions as in **Figure 2.**

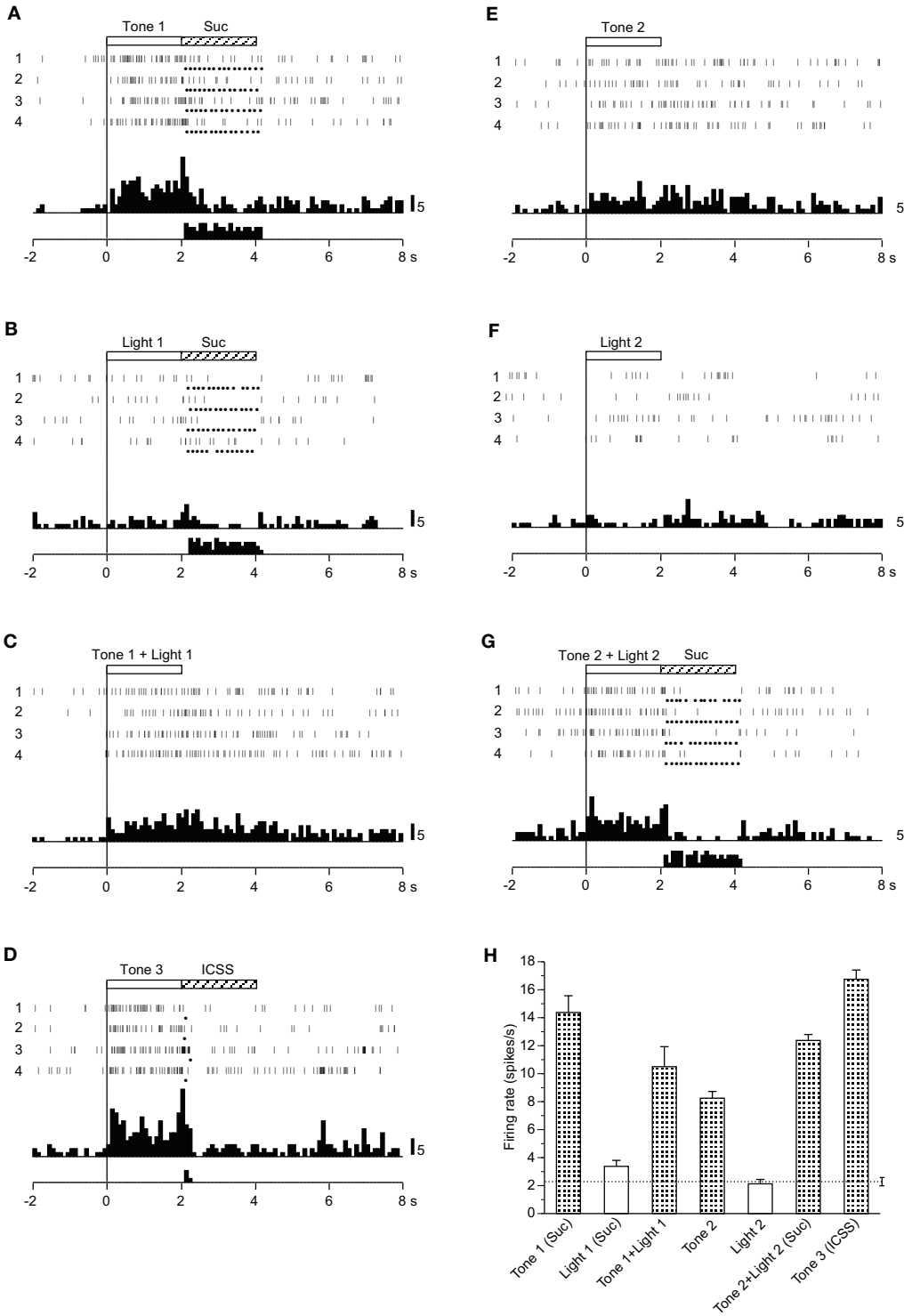

FIGURE 4 | Activity of a miscellaneous differential neuron that responded differently to the conditioned auditory stimuli. (A–G) Raster displays and histograms of neuronal responses to Tone 1 predicting the sucrose solution **(A)**, Light 1 predicting the sucrose solution **(B)**, Tone 1 + Light 1 predicting non-reward **(C)**, Tone 3 predicting intracranial self-stimulation (ICSS) **(D)**, Tone 2 predicting non-reward **(E)**, Light 2 predicting non-reward **(F)**, and Tone 2 + Light 2 predicting sucrose solution **(G)**. Note that neuronal activity increased during presentation of the CSs that included an auditory stimulus (i.e., Tone 1, Tone 1 + Light 1, Tone 3, Tone 2, and Tone 2 + Light 2) but not during presentation of a visual CS (Light 1 or Light 2). **(H)** Histogram of neuronal responses during presentation of each CS (mean firing rate ± SEM). Other descriptions as in **Figure 2.**

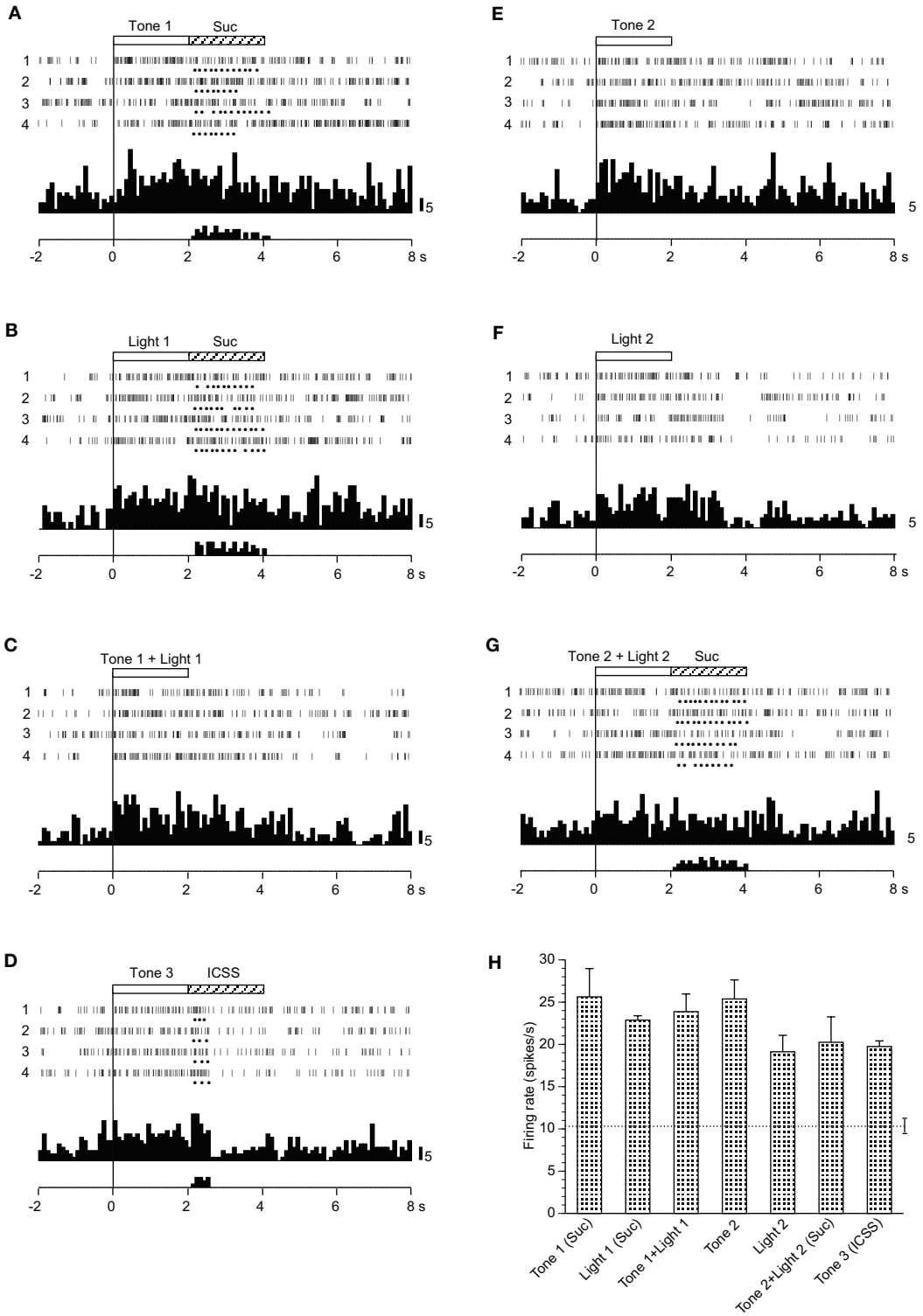

FIGURE 5 | Activity of a non-differential neuron that responded indiscriminately to the conditioned sensory stimuli. (A–G) Raster displays and histograms of neuronal responses to Tone 1 predicting the sucrose solution **(A)**, Light 1 predicting the sucrose solution **(B)**, Tone 1 + Light 1 predicting non-reward **(C)**, Tone 3 predicting intracranial self-stimulation (ICSS) **(D)**, Tone 2 predicting non-reward **(E)**, Light 2 predicting non-reward **(F)**, and Tone 2 + Light 2 predicting the sucrose solution **(G)**. Note that neuronal activity increased during presentation of all CSs. **(H)** Histogram of neuronal responses during presentation of each CS (mean firing rate ± SEM). Other descriptions as in **Figure 2.**

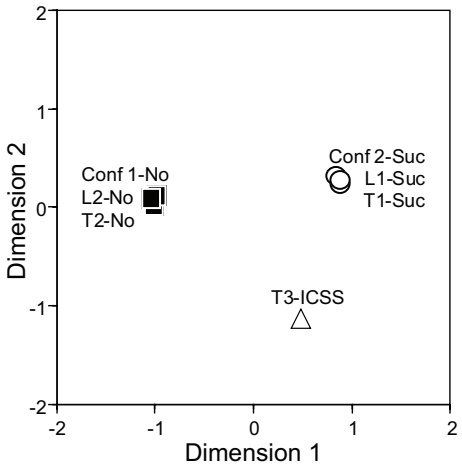

FIGURE 6 | Two-dimensional representation of the seven conditioned stimuli (CSs) resulting from multidimensional scaling using responses of 31 differential CS-related neurons. Note that three groups of CSs are recognized; CSs predicting the sucrose solution (Tone 1, Light 1, and Tone 2 + Light 2) indicated by white circles, the CS predicting intracranial self-stimulation (ICSS; Tone 3), indicated by a white triangle, and the CSs predicting non-reward (Tone 2, Light 2, Tone 1 + Light 1) indicated by black squares. Conf 1, Tone 1 + Light 1; Conf 2, Tone 2 + Light 2; L, Light; T, Tone.

Light 1, or Tone 2 + Light 2), (ii) CS predicting ICSS (Tone 3), and (iii) CSs predicting non-reward (Tone 2, Light 2, or Tone 1 + Light 1).

INGESTION/ICSS-RELATED NEURONS

Twenty-six neurons responded mainly during the reward phase (**Table 2**). A typical example of this type of neuron is shown in **Figure 7**. This neuron displayed excitatory responses during ingestion of a reward (sucrose solution and ICSS; **Figures 7A,B,D,G**). The activity of this neuron also increased in response to all CSs, except Light 2 (**Figures 7A–G**). The mean firing rates during presentation of all CSs except Light 2 and those during reward phases were significantly larger than those during the control phase ($p < 0.05$). The mean response magnitudes of the neuron during the CS and reward phases are presented in **Figure 7H**. Statistical comparisons indicated that significant differences occurred in the response magnitudes among the CSs [$F(6, 21) = 5.982$, $p < 0.01$], and in the response magnitudes among the reward phases [$F(3, 12) = 7.031$, $p < 0.01$]. Furthermore, the responses to the sucrose solution were significantly larger than those to ICSS ($p < 0.05$).

SPONTANEOUS FIRING RATE

The spontaneous firing rates of differential CS-related neurons ranged from 1.57 to 55.94 spikes/s (8.33 ± 2.07 spikes/s, mean ± SEM, $n = 31$); those of non-differential CS-related neurons ranged from 1.62 to 83.61 spikes/s (15.21 ± 6.10 spikes/s, $n = 15$); those of ingestion/ICSS-related neurons ranged from 1.05 to 85.61 spikes/s (13.02 ± 3.43 spikes/s, $n = 26$); and those of non-responsive neurons ranged from 0.26 to 78.07 spikes/s (11.86 ± 0.91 spikes/s, $n = 212$; **Figure 8A**). A statistical comparison by one-way ANOVA indicated that no significant differences in mean spontaneous firing rates were observed among these four types of neurons [$F(3, 280) = 0.970$, $p > 0.05$].

Significant differences were found for the mean spontaneous firing rates in septal neuron locations. Spontaneous firing rates of the LS, MS, and PS neurons ranged from 0.26 to 85.6 spikes/s (10.64 ± 0.84 spikes/s, $n = 249$), from 3.02 to 82.50 spikes/s (25.93 ± 3.75 spikes/s, $n = 24$), and from 2.22 to 9.49 spikes/s (5.95 ± 0.81 spikes/s, $n = 11$), respectively (**Figure 8B**). Statistical comparison by one-way ANOVA indicated a significant difference in mean spontaneous firing rates among the three regions [$F(2, 281) = 14.96$, $p < 0.01$]. The mean spontaneous firing rate of the MS neurons was significantly larger than that of the LS and PS neurons ($p < 0.01$).

DISTRIBUTIONS OF SEPTAL NEURON RECORDING SITES

The recording sites of all septal neurons are shown in **Figure 9**. Of 284 septal neurons recorded, 249 were located in the LS, 24 in the MS, and 11 in the PS. The distributions of each type of responsive neurons are shown in **Figure 10**. Differential CS-related neurons were located mainly in the LS ($n = 26$; excitation/inhibition: 18/8), and a few in the MS ($n = 2$; 0/2) and PS ($n = 3$; 2/1). Non-differential CS-related neurons were located only in the LS ($n = 15$; 7/8). Ingestion/ICSS-related neurons were located mainly in the LS ($n = 23$; 14/9) and a few in the MS ($n = 3$; 1/2). The responsive neurons in the LS tended to be located more densely in the intermediate and dorsal part of the LS.

DISCUSSION

Notably, most of the septal neurons were recorded from the LS, and only a few neurons were recorded from the MS and PS. Therefore, the following discussion is based largely on data from the LS.

DIFFERENTIAL CS-RELATED NEURONS

Previous unit recording studies in the MS and LS during classical conditioning reported that MS neurons display excitatory responses to a CS that predicts an aversive stimulus but display inhibitory responses to the CS predicting an appetitive stimulus (Yadin, 1989; Thomas et al., 1991). In contrast, LS neurons display inhibitory responses to the CS predicting an aversive stimulus and excitatory responses to the CS predicting an appetitive stimulus (Thomas et al., 1991). The present study was partially consistent with these results. Of 15 CS^+-related neurons in the LS, 11 showed excitatory responses, and two CS^+-related neurons in the MS showed inhibitory responses. These results are consistent with the idea that reciprocal relationships exist between LS and MS neurons. Neuroanatomical studies have reported that LS neurons receive excitatory glutamatergic afferents and send inhibitory gamma-aminobutyric acid (GABA)ergic efferents to the MS and lateral hypothalamic area (Panula et al., 1984; Risold and Swanson, 1997b), where inhibitory responses to the CS^+ have been reported (Ono et al., 1986; Yadin, 1989; Thomas et al., 1991; present results). This neurophysiological evidence along with neuroanatomical results indicating direct and indirect connections between the LS and MS (Risold and Swanson, 1997b) suggests that the LS and MS work as a functional unit during

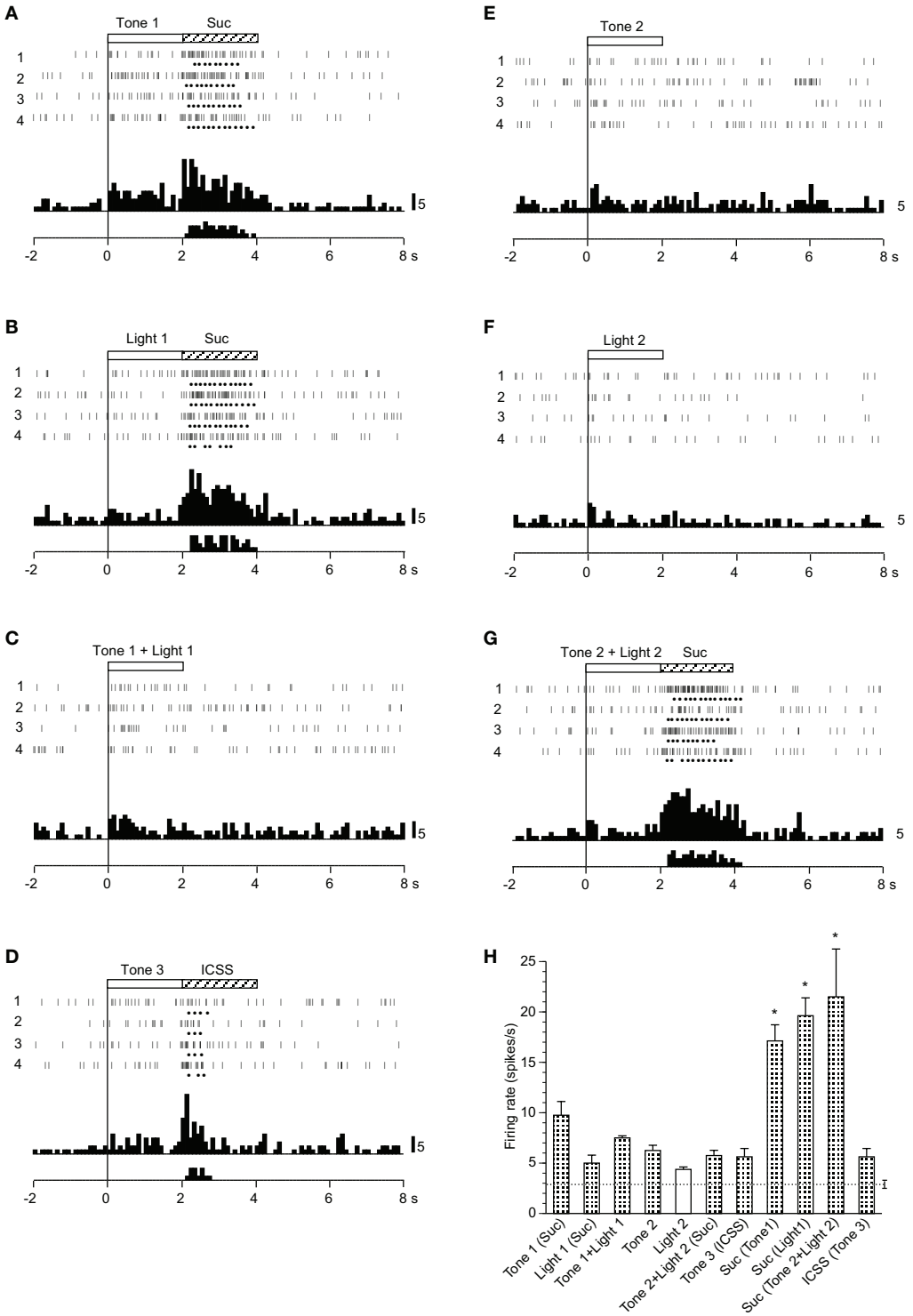

FIGURE 7 | Activity of an ingestion/ intracranial self-stimulation (ICSS)-related neuron that responded mainly during the reward phase. (A–G) Raster displays and histograms of neuronal responses to Tone 1 predicting sucrose solution **(A)**, Light 1 predicting sucrose solution **(B)**, Tone 1 + Light 1 predicting non-reward **(C)**, Tone 3 predicting ICSS **(D)**, Tone 2 predicting non-reward **(E)**, Light 2 predicting non-reward **(F)**, and Tone 2 + Light 2 predicting sucrose solution **(G)**. Note that neuronal activity increased during ingestion of a reward (i.e., sucrose solution or ICSS) and also during presentation of some CSs (Tone 1, Light 1, Tone 1 + Light 1, Tone 3, Tone 2, and Tone 2 + Light 2), but not Light 2. **(H)** Histogram of neuronal responses during presentation of each CS and ingestion of reward (mean firing rate ± SEM). *significant difference from other responses (new multiple range test after one-way ANOVA, $p < 0.05$). Other descriptions as in **Figure 2.**

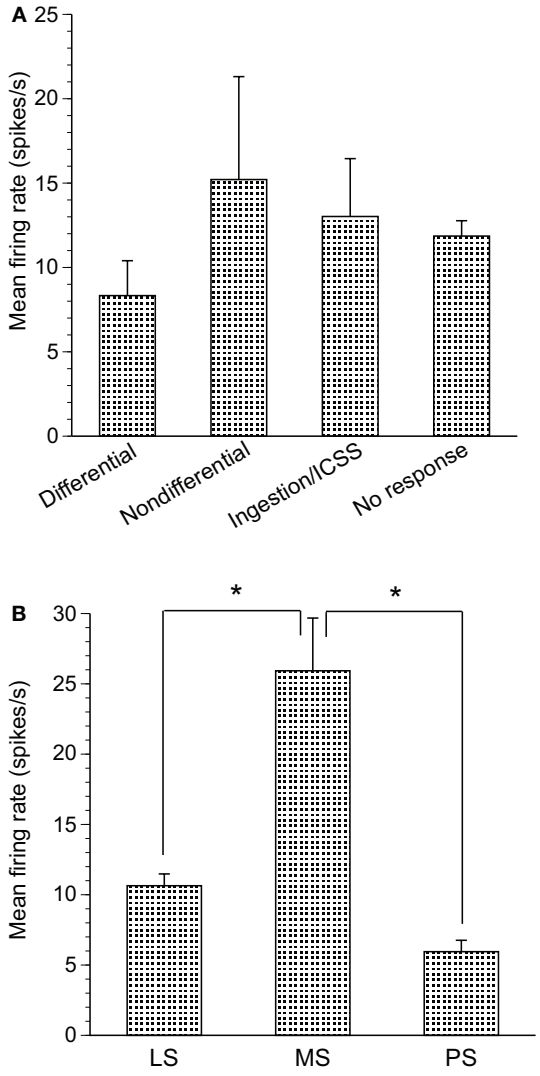

FIGURE 8 | Mean firing rates of neurons recorded in the septal nuclei. (A) The mean spontaneous firing rates (±SEM) of differential conditioned stimulus (CS)-related, non-differential CS-related, ingestion/intracranial self-stimulation (ICSS)-related and non-responsive neurons. No significant differences in mean spontaneous firing rates were found among the three groups (one-way ANOVA, $p > 0.05$). **(B)** The mean spontaneous firing rates (±SEM) of the lateral septal nucleus (LS), medial septal nucleus (MS), and posterior septal nucleus (PS) neurons. *significant difference in mean spontaneous firing rates (new multiple range test after a one-way ANOVA, $p < 0.05$).

conditioned associative learning. In contrast, four LS neurons showed inhibitory responses to the CS^+. A neuroanatomical study reported that LS neurons send their collaterals for intrinsic connections (Staiger and Nürnberger, 1991), which could induce inhibitory responses to CS^+ in the LS.

In the present study, four CS^0-related neurons were recorded from the LS. Similar CS^0-related neurons were previously reported only in monkey septal nuclei (Kita et al., 1995). Our previous studies indicated that no such neurons occur in the prefrontal area (Yamatani et al., 1990), cingulate cortex (Nishijo et al., 1997b; Takenouchi et al., 1999), amygdala (Nishijo et al., 1988a,b; Uwano et al., 1995), mediodorsal thalamic nucleus (Oyoshi et al., 1996), lateral hypothalamic area (Ono et al., 1986), and basal ganglia (Nishino et al., 1984, 1985a,b) in rats and monkeys. However, more recent studies in other laboratories have reported that some prefrontal cortical neurons predict the absence of reward (Kobayashi et al., 2002; Watanabe et al., 2002). Consistent with these results, the LS is positioned at an essential node point for integrating cognitive information from the various brain regions, including the prefrontal cortex, and relaying it to diencephalic and mesencephalic regions to directly control behavioral responses (Sheehan et al., 2004). Furthermore, the LS and MS have reciprocal connections with the nucleus of basalis of Meynert (substantia innominata; Lamour et al., 1984), which is involved in negative patterning similar to that (Tone1 + Light1) in the present study (Butt et al., 2002). Taken together, the existence of CS^0-related neurons in the LS suggests that cognitive information from the prefrontal cortex and the nucleus of basalis of Meynert may exert inhibitory control on emotional and motivational behaviors through the LS. Previous behavioral studies have consistently reported that electrically stimulating the septal area inhibits drinking (Wishart and Mogenson, 1970; Gordon and Johnson, 1981) and bar pressing for a food reward (Altman and Wishart, 1971) under a deprived condition.

In the present study, 18 CS^+-related and four CS^0-related neurons responded differently to the CSs in terms of reward contingency. For example, CS^+-related neurons responded to Tone 1 and Light 1 presented alone, which predicted reward, but not to these stimuli presented together, which predicted non-reward. On the contrary, CS^0-related neurons responded to Tone 1 and Light 1 presented together, but not to Tone 1 and Light 1 presented alone. It should be noted that, in the configural situation, the reward contingency of the stimuli presented together was opposite to that of the elemental stimuli presented alone, although the same sensory stimuli were involved. Therefore, these patterns strongly suggest that these responses to the CSs were related to the reward contingency of the CSs rather than to the physical properties of the stimuli. The results of the MDS support this idea. In two-dimensional space, seven CSs were categorized into the following three groups: (i) CSs predicting the sucrose solution (Tone 1, Light 1, or Tone 2 + Light 2), (ii) the CS predicting ICSS (Tone 3), and (iii) CSs predicting non-reward (Tone 2, Light 2, or Tone 1 + Light 1). Furthermore, the arrangement of these three groups of conditioned stimuli suggests that dimension 1 may reflect reward availability, whereas dimension 2 may reflect reward content (i.e., difference between sucrose and ICSS). This grouping of CSs based on reward contingency regardless of physical properties of the stimuli strongly suggests that reward contingency is a main determinant of LS neuronal responsiveness. This flexible encoding of reward contingency regardless of the physical properties of the CSs in the LS is reminiscent of the functions of both orbital and medial prefrontal cortices (flexible stimulus–reward associations; Jones and Mishkin, 1972; Ferry et al., 2000; McAlonan and Brown, 2003; Baxter et al., 2007; Tait and Brown, 2007; Hsu and Packard, 2008), from which the LS receives afferent inputs (Johnson et al., 1968; Sesack et al., 1989; Buchanan et al., 1994). These finding also suggest that the LS might be a key relay station for the prefrontal cortex to exert control over behavior.

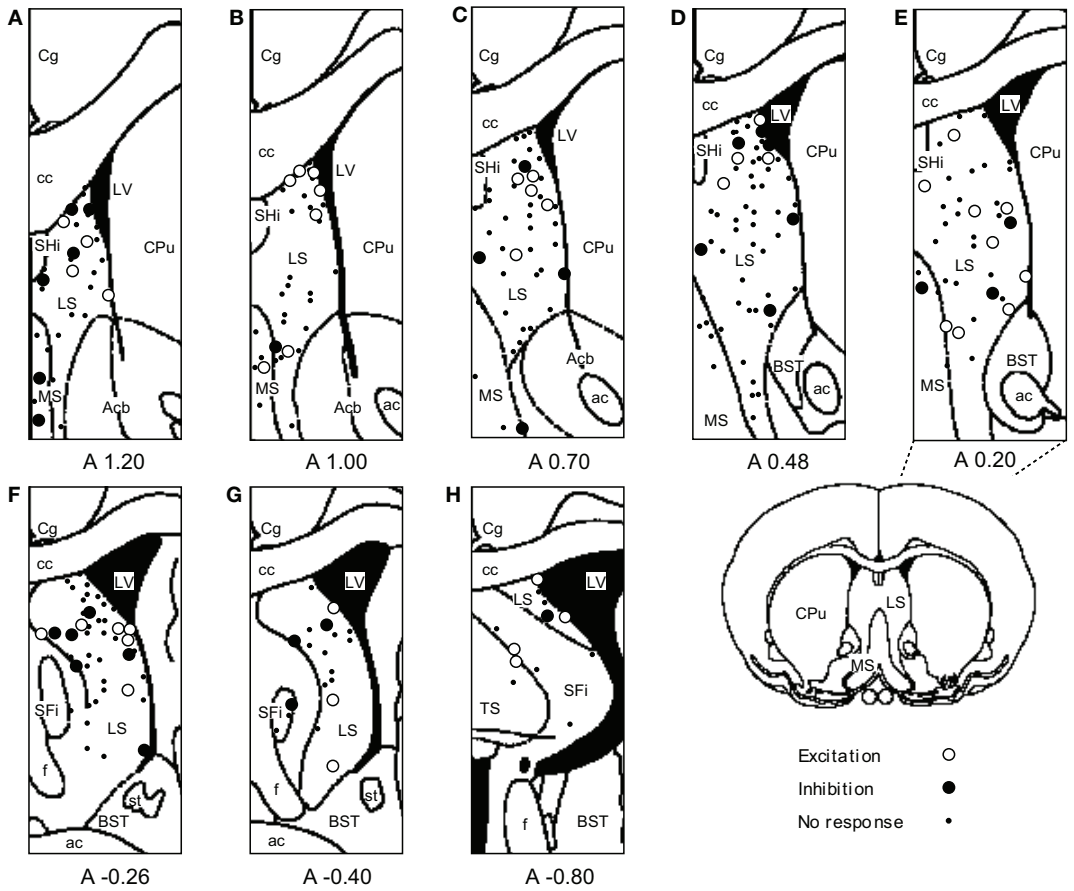

FIGURE 9 | Distributions of all neurons recorded from septal nuclei. (A–H) Frontal sections, based on the atlas of Paxinos and Watson, are arranged rostro-caudally from left to right. Values below each section indicate distance (mm) anterior (positive value) or posterior (negative value) from the bregma. White circles, excitation; black circles, inhibition; dots, no response. ac, anterior commissure; Acb, accumbens nucleus; BST, bed nucleus of the stria terminalis; cc, corpus callosum; Cg, cingulate cortex; CPu, caudate putamen; f, fornix; LS, lateral septal nucleus; LV, lateral ventricle; MS, medial septal nucleus; SFi, septofimbrial nucleus; SHi, septohippocampal nucleus; st, stria terminalis; TS, triangular septal nucleus.

NON-DIFFERENTIAL CS-RELATED NEURONS

The 15 non-differential CS-related neurons in the LS responded indiscriminately to all CSs regardless of reward contingency, suggesting that activity of non-differential CS-related neurons may reflect non-specific inputs such as arousal. The LS receives strong projections from brainstem nuclei such as the laterodorsal tegmental nucleus, locus coeruleus, raphe nucleus, and VTA, which are associated with modulating arousal level (Saper, 1987; Risold and Swanson, 1997b). Furthermore, the LS shares intimate connections with the lateral hypothalamic area (Jakab and Leranth, 1995), which composes a rostral portion of the ascending reticular formation (Nieuwenhuys et al., 1982). This evidence is consistent with a previous suggestion that septal nuclei are important for modulating hippocampal θ-rhythm, which is associated with behavioral arousal (Stewart and Vanderwolf, 1987a,b; Stewart and Steven, 1990).

INGESTION/ICSS-RELATED NEURONS

Fifteen ingestion/ICSS-related neurons responded mainly during ingestion of the reward. However, it is unclear whether the responses of these ingestion/ICSS-related neurons were related to reward ingestion or licking behavior, because EMGs were not monitored during recording of these neurons. However, activity of these neurons was not correlated to individual licks (data not shown). Furthermore, no neuronal activity changes were observed when spontaneous licking occurred during intertrial intervals. Additionally, about half of these neurons responded to both the sucrose solution and ICSS during the reward phases. These results suggest that the responses of ingestion/ICSS-related neurons are related to reward recognition. Similar ingestion-related neurons have been reported in monkey septal nuclei (Nishijo et al., 1997a). Activity of these neurons is modulated by satiation and correlated to the motivational level of the animal (Nishijo et al., 1997a) suggesting that septal nuclei play an important role in motivational aspects of ingestive behavior.

FUNCTIONAL CONSIDERATION OF THE SEPTAL NUCLEI

The LS consists of dorsal, intermediate, and ventral parts based on cytoarchitectonic parcelation (Swanson and Cowan, 1979). The LS receives major inputs from the hippocampal formation (Raisman, 1966a,b, 1969; Meibach and Siegel, 1977; Swanson and Cowan,

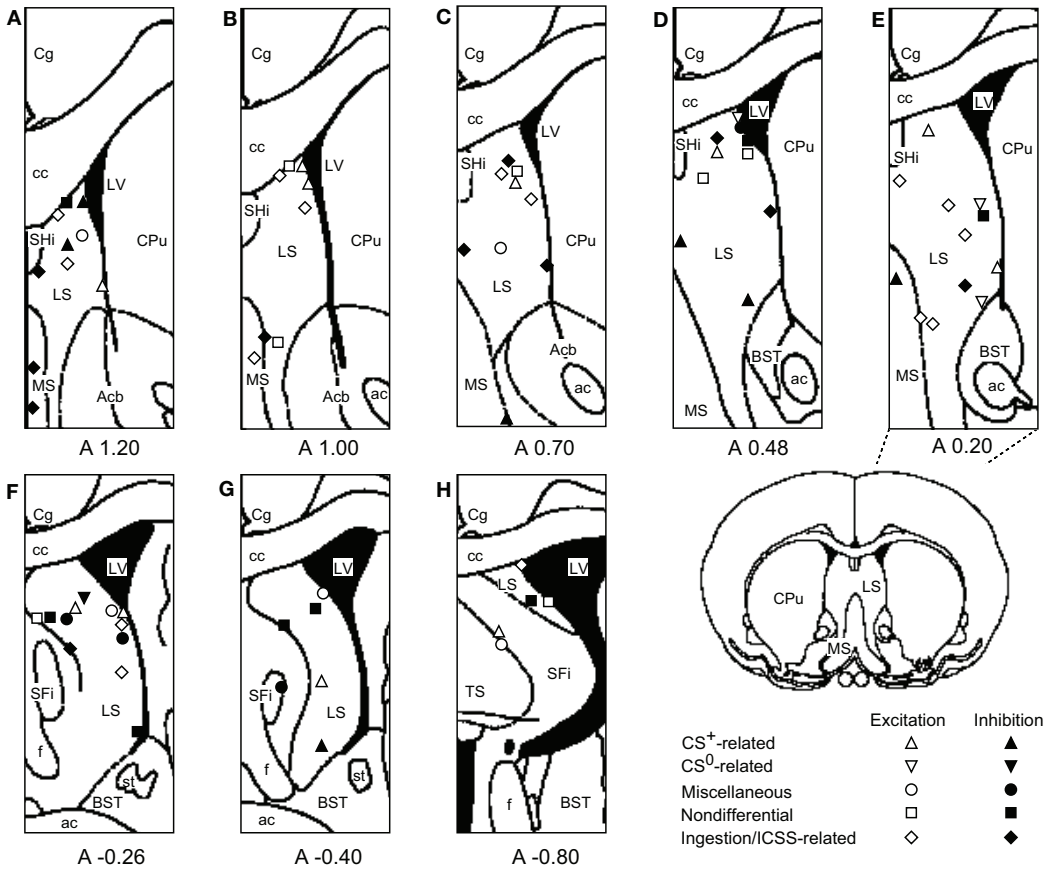

FIGURE 10 | Distributions of responsive septal neurons. (A-H) Frontal sections, based on the atlas of Paxinos and Watson, are arranged rostro-caudally from left to right. White symbols, excitation; black symbols, inhibition; triangles, conditioned stimulus predicting reward (CS^+)-related neurons; inverted triangles, conditioned stimulus predicting non-reward (CS^0)-related neurons; circles, miscellaneous differential neurons; squares, non-differential neurons; diamonds, ingestion/intracranial self-stimulation (ICSS)-related neurons. Other descriptions as in **Figure 9.**

1977, 1979), and the intermediate part of the LS receives most of the inputs (Staiger and Nürnberger, 1989). In the present study, most of the responsive neurons were located in the dorsal and intermediate part of the LS, and fewer neurons were located in the ventral part. The LS can also be divided into three parts based on chemoarchitecture (i.e., rostral, caudal, and ventral parts; Risold and Swanson, 1996, 1997a). The areas where responsive neurons were located (i.e., the dorsal and intermediate part of the LS by Swanson and Cowan, 1979) roughly corresponded to the caudal part of the LS and dorsolateral zone of the rostral part of the LS by Risold and Swanson (1996, 1997b), which receive afferents from the CA1 and CA3 subfields of the hippocampus as well as the subiculum. These results were consistent with a previous neurophysiological study in monkeys in which responsive septal neurons were located mainly in the same areas as those in the present study (Kita et al., 1995). Afferent fibers from the entorhinal cortex also terminate in the intermediate part of the LS (Alonso and Köhler, 1984). The bed nucleus of the stria terminalis projects to the intermediate and ventral part of the LS, and the medial amygdaloid nucleus projects to the ventral part of the LS (DeVries and Buijs, 1983; VanLeeuwen and Caffé, 1983; DeVries et al., 1985; Caffé et al., 1987; Mathieson et al., 1989; Staiger and Nürnberger, 1989; DeVries, 1990). The medial and orbital prefrontal cortex also project to both the LS and MS (Johnson et al., 1968; Sesack et al., 1989; Buchanan et al., 1994). These anatomical studies suggest that different information from the other limbic system noted above converges on the septal nuclei.

The major target of output fibers from the LS is the hypothalamus (Raisman, 1966a; Meibach and Siegel, 1977; Garris, 1979; Swanson and Cowan, 1979; Berk and Finkelstein, 1981; Sawchenko and Swanson, 1983; Wouterlood et al., 1988; Ferris et al., 1990; Staiger and Wouterlood, 1990; Staiger and Nürnberger, 1991; Risold and Swanson, 1996, 1997b). Electrically stimulating the septal nuclei may modulate motivated behaviors in which the hypothalamus plays an important role (Wishart and Mogenson, 1970; Altman and Wishart, 1971; Gordon and Johnson, 1981). Furthermore, lesions in the dorsal and intermediate parts of the septal nuclei, where most responsive neurons were located in the present study, induce anxiety and abnormal emotional behaviors in rats (Menard and Treit, 1995), and the antidepressant-like effects of allopregnanolone (GABAA receptor agonist) are mediated through its effects on the dorsal or intermediate part of the LS (Rodríguez-Landa et al., 2009). These behavioral changes after septal lesions and pharmacological stimulation may be related to a disconnection between the LS and the hypothalamus, and enhance LS control on the hypothalamus,

respectively. Additionally, the LS projects to the VTA to regulate dopaminergic neuron firing rates (see review by Sheehan et al., 2004). Taken together, the results suggest that the LS neurons receive a variety of information related to cognition, memory, and emotion from other limbic areas such as the hippocampal formation, entorhinal cortex, and amygdala, and integrate this information to control the diencephalon and brainstem including the hypothalamus, which play an important role in ingestive, motivated, and emotional behaviors.

CONCLUSION

The present study analyzed rat septal neuronal activity during discrimination of elemental and configural stimuli associated with reward or non-reward, and septal neurons were grouped into five types; (1) CS^+-related neurons responding selectively to the CSs predicting reward, (2) CS^0-related neurons responding to the CSs predicting non-reward, (3) neurons responding to some CSs predicting reward or non-reward, (4) septal neurons responding non-differentially to all CSs, and (5) septal neurons responding mainly during the ingestion/ICSS phase.

Multivariate analysis of the septal neuronal responses indicated that septal neurons represented the CSs based on reward contingency regardless of the physical properties of the stimuli and categorized them into three groups; three CSs predicting the sucrose solution, three CSs predicting non-reward, and one CS predicting the ICSS. These results along with those of previous anatomical studies suggest that septal nuclei integrate convergent environmental stimuli to represent reward contingency, which is essential to manifest appropriate behaviors in response to changing environmental stimuli.

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Electroencephalographic brain dynamics of memory encoding in emotionally arousing context

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Emotional content/context enhances declarative memory through modulation of encoding and retrieval mechanisms. At encoding, neurophysiological data have consistently demonstrated the subsequent memory effect in theta and gamma oscillations. Yet, the existing studies were focused on the emotional content effect and let the emotional context effect unexplored. We hypothesized that theta and gamma oscillations show higher evoked/induced activity during the encoding of visual stimuli when delivered in an emotionally arousing context. Twenty-five healthy volunteers underwent evoked potentials (EP) recordings using a 21 scalp electrodes montage. They attended to an audiovisual test of emotional declarative memory being randomly assigned to either emotionally arousing or neutral context. Visual stimulus presentation was used as the time-locking event. Grand-averages of the EP and evoked spectral perturbations were calculated for each volunteer. EP showed a higher negative deflection from 80 to 140 ms for the emotional condition. Such effect was observed over central, frontal and prefrontal locations bilaterally. Evoked theta power was higher in left parietal, central, frontal, and prefrontal electrodes from –50 to 300 ms in the emotional condition. Evoked gamma power was higher in the emotional condition with a spatial distribution that overlapped at some points with the theta topography. The early theta power increase could be related to expectancy induced by auditory information processing that facilitates visual encoding in emotional contexts. Together, our results suggest that declarative memory enhancement for both emotional content and emotional context are supported by similar neural mechanisms at encoding, and offer new evidence about the brain processing of relevant environmental stimuli.

Keywords: brain dynamics, declarative memory, emotionally arousing context, evoked potentials, evoked spectral perturbations

INTRODUCTION

Emotionally arousing events are better remembered than neutral ones (Dolan, 2002; LaBar and Cabeza, 2006; McGaugh, 2006). If someone is asked about what they were doing when they found out about the death of a loved relative, everyone remembers about the central fact (the notice), but also whom they were with, where, and what were they doing in that moment. Such clear evidence, from the everyday experience, demonstrates the effect of emotion over memory. Both the content of the information and the context in which the information is embedded enhance memory formation (LaBar and Cabeza, 2006; Barrett et al., 2007; Pastor et al., 2008). Controlled experiments in laboratory settings reinforce this idea and allow studying the mechanisms and neural structures involved in this phenomenon.

Functional neuroimaging and electroencephalographic (EEG) recordings during the encoding of the stimuli have revealed some brain structures and mechanisms that are critical for the episodic memory formation. Although some evidence do not agree, the consensus is that during the encoding of episodic memories the activity of left hemisphere is increased, especially at the medial temporal lobe, the parietal lobe, and the prefrontal cortex (Sederberg et al., 2003, 2007; Osipova et al., 2006).

Consistent evidence demonstrate that EEG theta and gamma oscillations are increased during the encoding of stimuli that will be remembered, if compared to stimuli that will be forgotten (Sederberg et al., 2003; Axmacher et al., 2006; Osipova et al., 2006; Guderian et al., 2009; Duzel et al., 2010; Jutras and Buffalo, 2010). This is the so called subsequent memory effect. Alpha oscillations have been studied also, however, there is less consensus about their role during memory encoding (Klimesch, 1997, 1999; Klimesch et al., 2000, 2005). The evoked and induced activities measured by the event-related potentials technique have shown even less agreement about the time windows and the source of the activity (Hillyard and Anllo-Vento, 1998; Vogel and Luck, 2000; Herrmann and Knight, 2001; Schupp et al., 2007). Nevertheless, methodological variances such as the kind of stimuli, the tasks used, and the type of measures extracted from the raw data might account for such divergence (Klimesch et al., 2000; Herrmann and Knight, 2001).

Some studies explored the effect of emotion over encoding and demonstrated a privileged access to neural processing resources that could lead to better memory formation (Bradley et al., 2001; Foti et al., 2009; Weinberg and Hajcak, 2010). They focused on the effect of the emotional content of visual stimuli, and found that the evoked and induced activities are modulated by the characteristics of the pictures. In one study, the effect of the emotional context over the processing of visual stimuli was assessed by the event-related potentials (ERP) technique (Pastor et al., 2008). They found that neutral stimuli induced higher late positive potentials (400–700 ms) when presented within an emotional context when

compared to a mixed context. However, in this study the emotional context was created by the same visual stimuli when they were presented in blocks of stimuli with the same emotional valence. Nevertheless, this study did not test if the context exerted any effect over memory formation (subsequent memory effect). Moreover, the analyses applied in the above mentioned studies explored the effects of emotion using only time-domain techniques. Meanwhile, time–frequency decompositions might better describe these effects.

Our group has extensively used an emotional memory test (Cahill and McGaugh, 1995) in which visual and auditory information is delivered simultaneously (Frank and Tomaz, 2000; Gasbarri et al., 2005, 2006; Uribe et al., 2008). With this methodology we demonstrated that the emotional content enhances the episodic memory formation. However, the contribution of visual and auditory information in the memory facilitation could not be differentiated. In the present study we modified this methodology in order to explore the processing of visual information within the emotional context created by the narrative (Gasbarri et al., 2006). For this purpose we employed EEG recordings during the presentation of an emotional memory test in which the narrative preceded, and was not concomitant with, the visual stimuli presentation. We analyzed the brain activity related to visual stimuli processing through time-domain and frequency-domain methods.

We hypothesize that the auditory emotional context will enhance the episodic memory for the visual stimuli. This enhancement will be achieved through mechanisms similar to those present in the processing of stimuli with emotional content. Additionally, we hypothesize that oscillatory activity measured by time–frequency decompositions will be better suited to analyze the brain activity related to encoding of stimuli.

METHODOLOGY

SUBJECTS

Twenty-five right-handed volunteers [age 23.3 (SEM=0.9); 12 female] were recruited through announcement in the campus. None had antecedents of neurologic or psychiatric illnesses, concomitant use of medications or acoustic impair. Beck Depression Inventory and Beck Anxiety Inventory were applied to each volunteer. Scores of every volunteer were within the range expected in the normal Brazilian population (Gorenstein and Andrade, 1996) and none met the exclusion criteria. Written informed consent was obtained from each volunteer prior to any procedure. They did not receive money or other incentive for their participation. Volunteers were naïve about the aims of the study; however, at the end of the procedure, researchers explained to them the real objectives of the study. The experimental protocol was approved by institutional ethics committee.

EMOTIONAL MEMORY TEST

A series of 11 slides accompanied by a narration were used as stimuli. Slide consisted in the same material used by Frank and Tomaz (2000). Two different narrations could accompany these slides, both of them narrated by the same person with an unemotional voice and with a similar grammatical construction in both versions. The narrative is the same for both versions during the first four slides (Phase 1) and differs between versions during the remaining seven slides (Phase 2 and 3). The second part of the narrative (Phase 2 and 3) has emotionally neutral content in the Neutral (N) version and emotionally arousing content in the Emotional (E) version. A detailed description of the test could be found in Frank and Tomaz (2000). Narratives were delivered through headphones. In order to avoid concurrent processing of visual and audio information, narratives were delivered during the fixation cross images between the slides. Because an ERP study requires a minimal number of trials, each slide was presented twice (**Figure 1**). After the slide presentation, subjects were asked to score the story using the Self Assessment Manikin (SAM; Bradley and Lang, 1994), for both valence and arousal dimensions. One week later, the volunteers returned and performed a retrieval task in which they had to write down all the information they could remember about the slides and the narrative presented during the past session.

EEG RECORDINGS

EEG data were recorded using a NeuronSpectrum 4EP system (Neurosoft, Russia) at a sampling rate of 1000 Hz and with a 0.5–75 Hz band-pass filter. Twenty-one scalp electrodes (10–20 system) referenced to linked mastoids recorded the cortical activity of each volunteer. Each local of the electrodes was cleaned and prepared by gently rubbing with an abrasive gel (Nuprep, Weaver and Company, USA). A conductive paste (Ten20, Weaver and Company, USA) fixed the electrodes and maintained all the impedances below 5 kΩ during the whole session.

PROCEDURE

Volunteers were received in the recording room. They read and signed the written informed consent and responded to a brief interview before responding to the two inventories. Slides were presented in a 17′ flat screen at approximately 60 cm from the subject. They sat comfortably in front of the screen while the electrodes and the

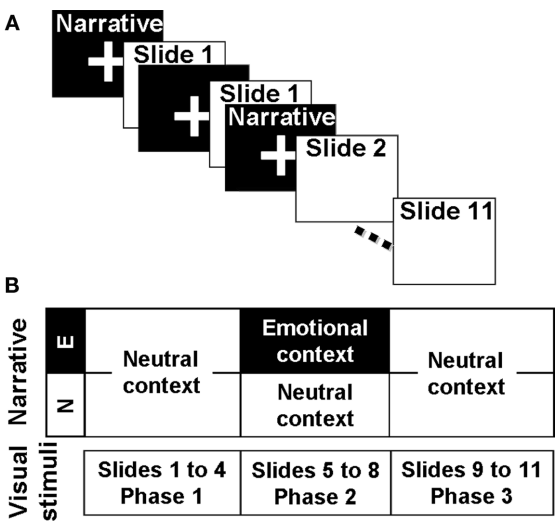

FIGURE 1 | Schematic representation of the experimental procedure. (A) Slide presentation sequence. Narrative was not concomitant with the slide presentation and preceded the image of the scene it describes. As shown, each slide was presented twice. **(B)** Auditory and visual material. Images were the same for both versions of the test and were grouped in three phases. The narrative that accompanied the Phase 2 was different for each version.

headphones were installed. The researcher asked to stay as quite as possible in order to avoid artifacts and to attend to both slides and narrative as they attend to a TV show. After the slide presentation, volunteers responded to the SAM and left the room. One week after, they returned to perform the retrieval task. Luminance, audio volume and instructions were kept constant.

DATA PROCESSING

All data processing and analyses were performed using EEGLAB v9.0.0.0b a freely available open source toolbox (http://sccn.ucsd.edu/eeglab/) suited for EEG data management under Matlab® or Octave platform (Delorme and Makeig, 2004; Makeig et al., 2004).

ICA DECOMPOSITION

Standard channel coordinates of the BEM dipfit model were used for all the electrodes. ICA decomposition was performed using the Infomax (*runica*) algorithm (Bell and Sejnowski, 1995). Both Rest (Phase 1) and Test (Phase 2) periods were submitted to the same analysis as concatenated datasets. EEG recordings were decomposed in 21 independent components (IC). Eye blink and saccadic movements (from the Test period) were always represented by two or three IC which were selected and eliminated in both rest and test datasets. The back-projection of the remaining IC resulted in the "clean datasets" used for all the analyses.

EVOKED POTENTIALS (EP) AND EVOKED SPECTRAL PERTURBATIONS (ESP)

In some cases, differences in the mean average evoked activity (time domain) were not found, while differences in oscillatory activity (frequency domain) appeared (Klimesch et al., 2000; Makeig et al., 2004; Osipova et al., 2006).

Events were recorded in a separate channel during the Test period and then extracted to set the time 0 for each epoch. Mean baseline (−1000 to 0 ms) was subtracted from each epoch. Several methods to reject bad epochs were applied to the 21 channels. All epochs with extreme values (higher than |75| μV), abnormal trend (slope higher than 50 μV/epoch and R^2 higher than 0.3), improbable data (data beyond 4 SD in a single channel or all channels), abnormal data distribution (any epoch with data distribution beyond 5 SD) or abnormal spectrum (higher than |25| dB in the 0–2 Hz range) were automatically rejected. The remaining epochs were used in the EP and ESP analyses. EP grand-average was calculated for each volunteer. ESP images were computed based on 10 trials of 3000 frames using three cycles at the lowest frequency and 250 cycles at the highest. Frequency resolution was set to 100 log spaced points from 3 to 500 Hz.

STATISTICAL ANALYSES

Electrophysiology and neuroimaging studies use the subsequent memory effect to point out which measures can be associated with encoding success (Sederberg et al., 2003; Osipova et al., 2006; Jutras and Buffalo, 2010; Nyhus and Curran, 2010). This can be done by comparing the brain activity at the encoding of an item that is successfully retrieved or recognized in a later session against the activity at the encoding of an item that is not retrieved/recognized. On the other hand, we classified the items based on the context they were embedded as previous studies did (Cahill and McGaugh, 1995; Frank and Tomaz, 2000; Gasbarri et al., 2005; Uribe et al., 2008).

Despite the present work did not select the items based on individual retrieval, the methodology is compatible with the subsequent memory effect.

Independent-samples t-tests compared age, arousal, and valence between groups. Retrieval scores were divided according to the phase the information belongs to. These scores were analyzed by a mixed design ANOVA with the factors Group (Neutral or Emotional; between-subjects) and Phase (1 and 2; within-subjects). *Post hoc* analyses were performed with independent-samples t-tests. Significance level for multiple comparisons was adjusted with Bonferroni method. Analyses of the EEG data (EP and ESP) were performed with the parametric statistical tools provided by the EEGLAB platform. Only data from Phase 1 and Phase 2 were considered, because they best represent baseline and experimental conditions. Results are expressed as mean (SEM).

RESULTS

BEHAVIORAL DATA

Mean age were not different between the Emotional (E) and Neutral (N) groups [21.7 (0.7) and 24.8 (1.6) respectively; $p = 0.062$]. As expected, the mean valence score was lower and the mean arousal score was higher in the E group. Total retrieval, as well as Phase 1 and Phase 3 retrieval, was not different between groups. However, mean score of Phase 2 retrieval was higher in the E group (**Table 1**).

EVOKED POTENTIALS

Grand-average potential related to Phase 2 slides differed between groups around 120 ms (from 80 to 140 ms) after the stimuli onset (**Figure 2A**). The anterior (frontal and central) electrodes registered a more negative potential in the E group during this time interval (**Figure 2B**). There were some spurious differences between groups at later times but they were not sustained over time and lasted no more than 10 ms.

OSCILLATORY ACTIVITY – EVENT-RELATED SPECTRAL PERTURBATIONS

Theta band (4–7 Hz)

Prefrontal (Fp1, Fpz, Fp2), frontal (F7, F3, Fz, F4, F8), central (C3, Cz, C4), and left parietal (P3, Pz) electrodes showed higher theta-band power in the E group than in the N group at the onset of the Phase 2 slides. In some cases, such differences were present even before the stimuli onset (−50 ms). Theta-band power remained higher after 300 ms from the stimuli onset in prefrontal, left frontal, left central, and midline parietal electrodes (**Figure 3**).

Table 1 | Mean (SEM) scores of behavioral data for each group.

	Neutral	Emotional	*p*-value
Valence	5.2 (0.4)	2.3 (0.4)	<0.001
Arousal	2.8 (0.8)	5 (0.9)	0.041
RETRIEVAL			
Total	15.2 (2.1)	12.6 (0.9)	0.138
Phase 1	4.7 (0.6)	4.8 (0.6)	0.853
Phase 2	2.5 (0.5)	6.2 (0.5)	<0.001
Phase 3	4.3 (0.7)	3.8 (0.7)	0.303

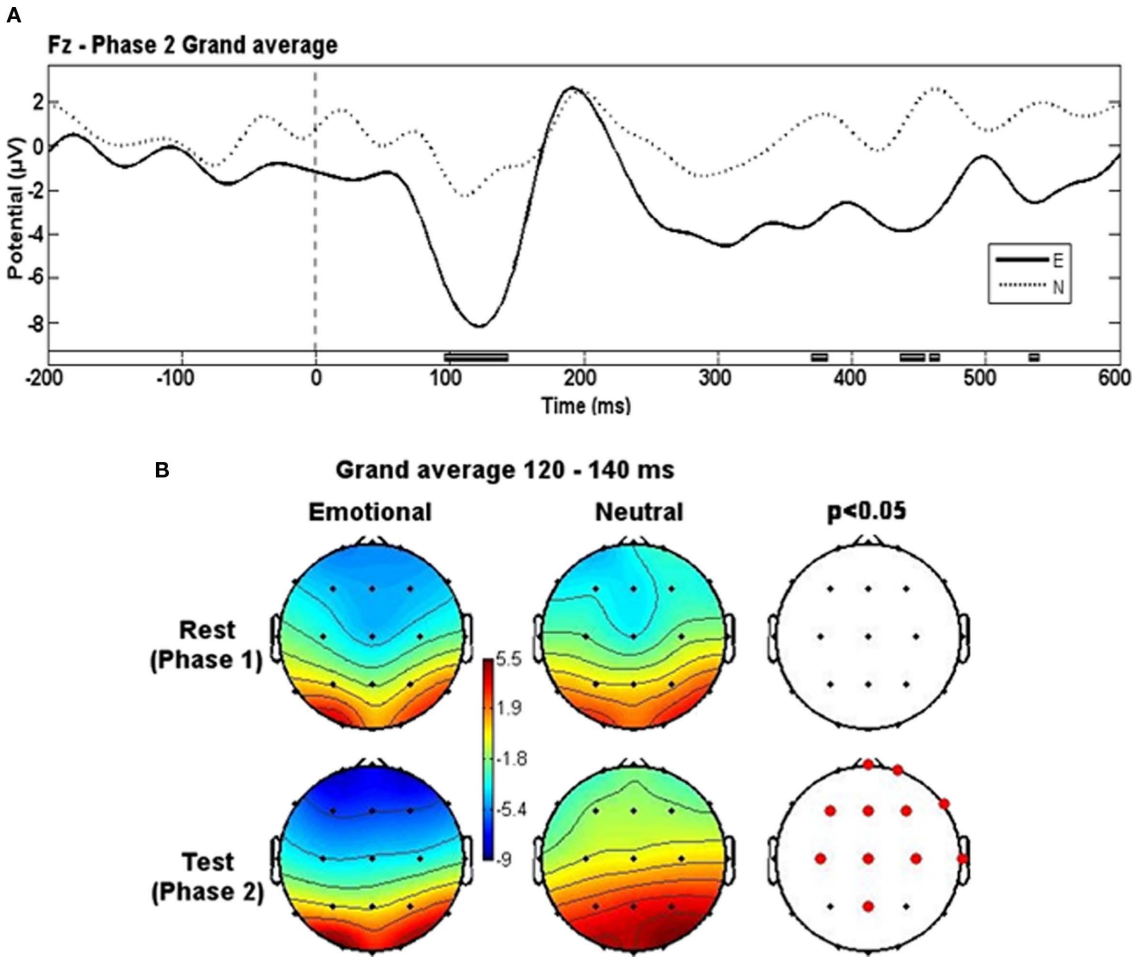

FIGURE 2 | Evoked potentials grand-averages. (A) Phase 2 grand-averages for E and N groups. Black blocks at bottom represent the time intervals where significant differences appeared. Low-pass filter of 20 Hz. (**B**) Topographical representation of activity for each group in both phases at the 120–140 ms time window. Red dots represent electrode locations where significant differences appeared.

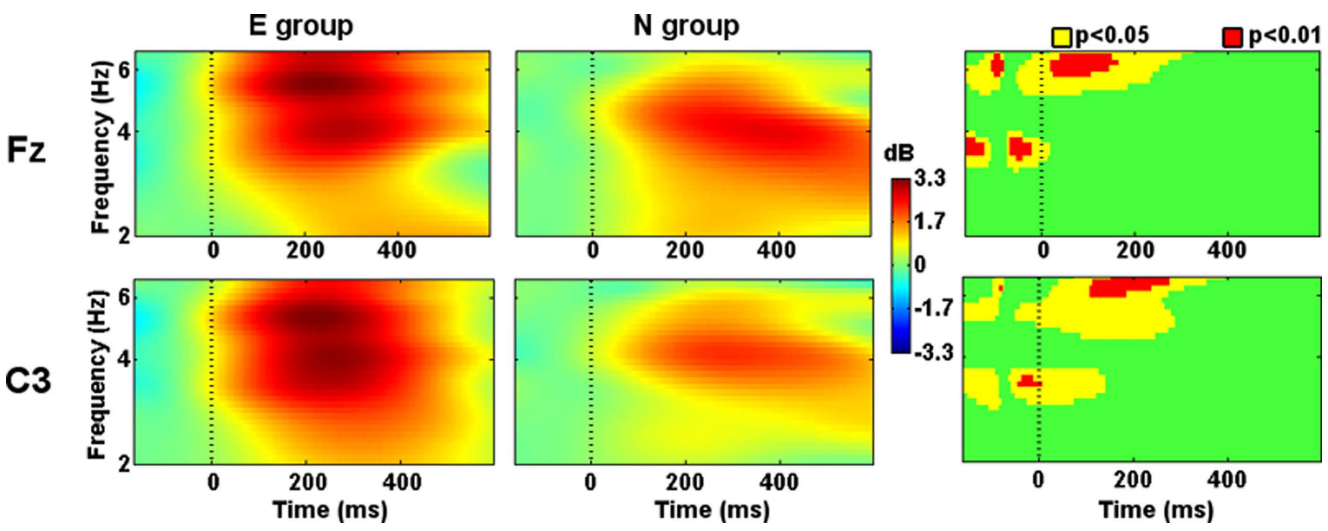

FIGURE 3 | Evoked spectral perturbations in theta band. Activity recorded from Fz and C3 during Phase 2 stimuli presentation.

Alpha band (7–13 Hz)

Power within 10–12 Hz was higher in the E group than in the N group at right parietal (P4) scalp location from −50 ms to 400 ms after the Phase 2 stimuli onset (**Figure 4**). This differential activity was not stationary and showed some changes. At higher latencies, the frequency where such differential activity appeared was faster.

Gamma band (30–50 Hz)

Since this frequency band is wide, we decided to split it in Gamma1 (≤40 Hz) and Gamma2 (>40 Hz) in order to facilitate the description and discussion of the results. Gamma1 showed higher activity in the N group from −50 to 400 ms after the Phase 2 stimuli onset in the T6 location (**Figure 5**). Around 300 ms left prefrontal (Fp1), right central (C4), left temporal (T3), and occipital locations (O1, Oz, and O2) showed increased activity in the N group too. In T3 and C4 (**Figure 5**), such activity was sustained until 500 ms after the stimuli onset.

Gamma2 showed higher activity in the E group from 0 to 400 ms in the F7 location (**Figure 5**). From 300 to 400 ms left parietal (P3) showed higher activity in the E group and, right central (C4) electrode showed higher activity in the N group (**Figure 5**). From 400 to 500 ms right occipital (Oz, O2) and

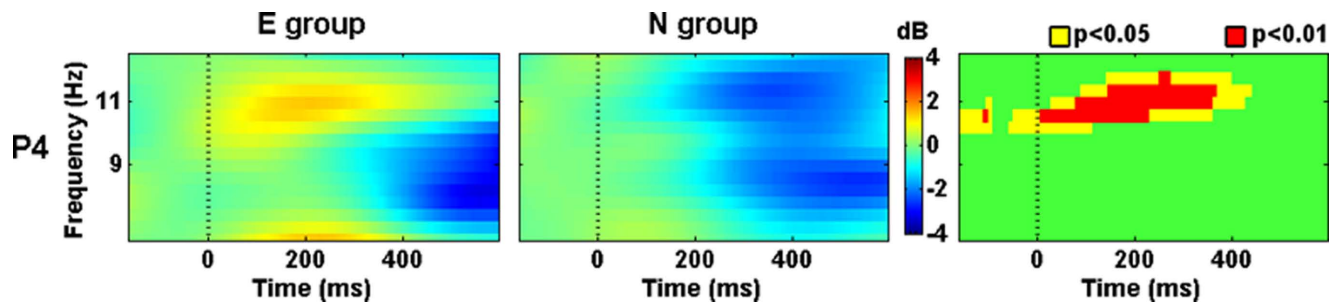

FIGURE 4 | Evoked spectral perturbations in alpha band. Activity recorded from P4 during Phase 2 stimuli presentation.

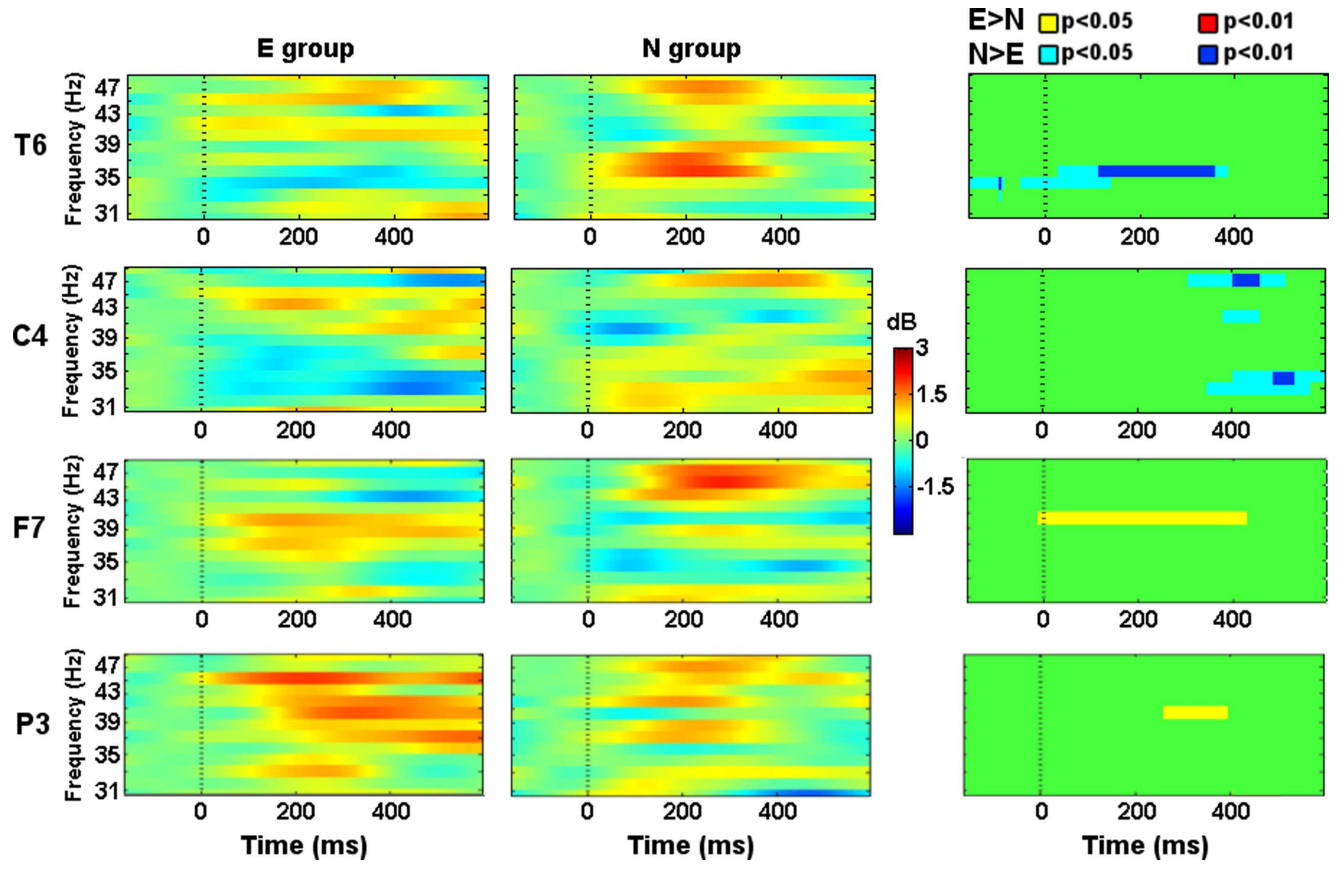

FIGURE 5 | Evoked spectral perturbations in gamma band. Activity recorded from T6, C4, F7, and P3 during Phase 2 stimuli presentation.

parietal (P4) showed higher activity in the E group and, prefrontal (Fp1, Fpz, Fp2) electrodes showed higher activity in the N group.

DISCUSSION

This study explored the brain dynamics, through EP and ESP, during the encoding of visual stimuli in either emotionally arousing or neutral contexts.

AROUSAL, VALENCE AND RETRIEVAL

Behavioral data replicated previous results that demonstrated higher arousal and lower valence scores for the emotional version of this test (Cahill and McGaugh, 1995; Frank and Tomaz, 2000; Gasbarri et al., 2005; Uribe et al., 2008). These results ensure that each group was exposed to the same visual stimuli but in a diverse context. Hence, we can state that any difference in memory performance and in brain activity can be attributed to the emotional context.

Regarding retrieval, our results showed a memory enhancement for items related to the second phase. In line with previous studies (Cahill and McGaugh, 1995; Frank and Tomaz, 2000; Gasbarri et al., 2005), the effect over declarative memory is limited to Phase 2 of the emotional version of this test, in which emotion is delivered.

EVOKED POTENTIALS

Early EP (P1 and N1) have been related to sensory processes and attention (Luck et al., 1990; Hillyard and Anllo-Vento, 1998; Vogel and Luck, 2000). In both auditory and visual domains, it is assumed that these potentials are modulated by the physical characteristics of the stimuli and not by cognition. Some authors differentiate the attentional effect over each potential and suggest that P1 represents the facilitation of the sensory processing for stimuli presented at an already attended location (spatial selective). On the other hand, N1 might represent the orienting response toward task-relevant stimuli (object selective; Luck et al., 1990; Hillyard and Anllo-Vento, 1998; Herrmann and Knight, 2001; Beaucousin et al., 2011). However, some authors argue that N1 is also modulated by previous experience and by the semantic category of the stimuli configuring the called "initial visual categorization" phenomenon (Bentin and Golland, 2002).

Emotional stimuli (both appetitive and aversive) capture attention (Bradley et al., 2001), and attention is also voluntarily directed to them (Schupp et al., 2007). Several studies showed greater negativities in early time windows (N1) for emotional compared to neutral stimuli (Schupp et al., 2007; Foti et al., 2009; Weinberg and Hajcak, 2010) reflecting such preferential processing and the initial visual categorization phenomenon.

The results of the present study showed that emotional context modified the brain activity (deeper negativity) evoked from 80 to 140 ms after visual stimuli onset. Nevertheless, pictures in both versions of the test were the same. The differential processing of visual information can only be attributed to the emotional context created by the auditory information. This statement is in agreement with the initial visual categorization phenomenon if we consider that identical images can be interpreted differently depending on their context.

The attention effect over N1 commonly shows a posterior distribution (central, parietal, and occipital; Vogel and Luck, 2000; Weinberg and Hajcak, 2010), but anterior distribution (central and frontal) has been found too (Vogel and Luck, 2000; Töllner et al., 2009). In one study using simple or choice-reaction time to visual stimuli, the anterior N1 found was actually a brain potential related to pre-motor activity or preparatory processes (Vogel and Luck, 2000). The predictable timing of the pictures in the task was responsible, since an unpredictable onset of the stimuli suppressed the anterior N1. Although in the present study the visual stimuli onsets were predictable, subjects did not perform any motor response. Then, the results suggest that other preparatory processes (e.g., expectancy, arousal) might be increased by an emotional context.

Yet, another study considered the anterior N1 as the neural index of attention during modality shifting (Töllner et al., 2009). It demonstrated that the amplitude of the anterior N1 component was higher when the target modality (visual or tactile) changed between trials. It also ruled out any contribution of response-related factors. In the present study, the first of the two appearances of each picture was preceded by the narrative in both versions. If N1 is due only to modality shifting, differences between groups might not appear. If the modality shifting effect was present in one half of the stimuli, we can conclude that the emotional context boosted this effect. Interestingly, the anterior N1 of the modality shifting effect (Töllner et al., 2009) had a left lateralized distribution similar to the results of the present study.

OSCILLATORY ACTIVITY

Growing evidence demonstrates that modulation of the oscillatory brain activity is related to successful memory encoding (Klimesch et al., 1997b; Axmacher et al., 2006; Osipova et al., 2006; Jutras and Buffalo, 2010; Nyhus and Curran, 2010). The results of the present work are consistent with previous studies and demonstrated that changes in theta, alpha, and gamma bands enhance declarative memory.

THETA OSCILLATIONS

The amplitude of theta oscillations in the medial temporal lobe predicted the successful encoding of the stimuli (Klimesch et al., 1997b; Klimesch, 1999; Osipova et al., 2006). If the timing of stimuli onset is predictable, theta oscillations are higher during the encoding of remembered items even before the stimuli onset (Sederberg et al., 2003, 2007; Guderian et al., 2009). This pre-stimuli theta level seems to be an active process that acts independently and in synergy with other cognitive mechanisms that facilitate encoding such as the level of processing, novelty expectancy, spatial location, etc. (Guderian et al., 2009).

Theta evoked and induced activity over the left hemisphere was higher in the E group. This wide spread activity is coherent with its role of communicating functionally linked networks from remote locations (Buzsaki and Draguhn, 2004; McCartney et al., 2004; Axmacher et al., 2006; Klimesch et al., 2008; Duzel et al., 2010; Nyhus and Curran, 2010). The stimuli in the present study had a predictable timing and the results showed an increase in theta oscillations even before the stimuli onset, preferentially at frontal locations. This is in line with the hypothesis

of top–down modulation of the encoding areas by the frontal executive cortex (Nyhus and Curran, 2010). Parietal and temporal theta oscillation might be related to stimuli processing in associative areas of the cortex and encoding in the structures responsible for the episodic memory (medial temporal lobe). However, the precise sources of activity could not be determined with this low-density array of electrodes. The present study design did not allow to discriminate the effects of novelty expectancy and level of processing over theta oscillations. However, we suggest that both of them should be present in an emotional context and that they will subsequently engage attention and enhance memory formation.

The time window of the N1 potential falls within the peak of theta oscillations increase and overlaps at some locations of the scalp. Interestingly, both theta oscillations and N1 have been related to the same processes (Luck et al., 1990; Klimesch et al., 1997b; Hillyard and Anllo-Vento, 1998; Klimesch, 1999; Bradley et al., 2001; Herrmann and Knight, 2001; Osipova et al., 2006; Beaucousin et al., 2011).

ALPHA OSCILLATIONS

Alpha power decrease has been extensively studied and consistently related to attention and memory encoding (Klimesch, 1997, 1999; Klimesch et al., 1997b, 2008). This alpha event related desynchronization (ERD) is accompanied by a reset of alpha phase that lead to an increase in the alpha evoked activity. This is called the alpha paradox (Klimesch et al., 1999, 2000; Herrmann and Knight, 2001).

In the extensive work about alpha ERD during memory tasks (preferentially semantic tasks) the called lower alpha (around 6–10 Hz) has been related to expectancy and attention, and showed a widespread distribution over the entire scalp. Note that the results of Theta oscillations (4–7 Hz) described before are in line with the results of the ERD literature. Meanwhile, upper alpha (around 10–12 Hz) showed a more restricted distribution (central and parietal locations) and has been related to semantic processing (Klimesch et al., 1997a,b, 2005; Klimesch, 1999).

The results of the present study demonstrated that evoked alpha oscillations (10–12 Hz) recorded at P4 were higher at the onset of the stimuli embedded in the emotional context. This frequency band is compatible with the called upper alpha. However, the contribution of semantic processing, that is suggested to be related to upper alpha ERD, has not been searched using this test of emotional memory. Anyway, since this is an episodic memory task, the influence of semantic processing should not be relevant. Additionally, since the ERD literature has focused on the semantic memory tasks it is possible that the encoding of the two declarative memory subtypes displays a hemisphere-specific pattern.

Although spectral perturbations and EP are obtained by different methodologies, both measures are related and could represent similar, or at least, complementary processes (Klimesch et al., 1999, 2000; Herrmann and Knight, 2001; Makeig et al., 2004). Noteworthy, such changes in the alpha oscillations were restricted to the same location in which the P300 event-related potential was increased during the encoding of the same emotional memory task (Gasbarri et al., 2006).

GAMMA OSCILLATIONS

Gamma oscillations seem to be involved in almost every class of sensory and cognitive functions (Klimesch et al., 2008). One study found higher induced occipital gamma activity during the encoding (0.3–1 s after stimuli onset) of later remembered pictures (Osipova et al., 2006). Noteworthy, the onset of the pictures in that study was not predictable. With predictable timing, on the other hand, evoked and induced gamma activity increases were related to successful encoding (Sederberg et al., 2003, 2007). The present study found that evoked (0–0.3 s) and induced (0.3–0.5 s) gamma activity present in different loci were higher for Phase 2 items. Evoked gamma activity has been related to expectancy and attention (Freunberger et al., 2007). This can be the case of the present study, because all the stimuli had a predictable onset. On the other hand, induced gamma activity is thought to reflect visual information processing (Freunberger et al., 2007). Gamma oscillations, both evoked and induced, showed different patterns of activity between E and N groups. As shown by a previous study using common nouns list, the subsequent memory effect can also be related to a decrease of the low gamma activity (<42 Hz) that coexists with gamma activity increase at other locations (Sederberg et al., 2007). Alternatively, our results might be a consequence of the different context that confers diverse meanings to the same picture. It is reasonable to assume that the oriented attention and the perceived characteristics of the stimuli differed between groups. Those differences might account for the frequency, temporal and spatial differences in the gamma activity.

THETA AND GAMMA INTERACTION

Theta activity seems to facilitate communication between distant neural assemblies while gamma activity seems to reflect short-distance synchrony (Buzsaki and Draguhn, 2004; Axmacher et al., 2006; Jutras and Buffalo, 2010; Nyhus and Curran, 2010). Both rhythms are intimately associated and their interaction might create the conditions necessary to long-term hippocampus-dependent memory formation (McCartney et al., 2004; Jutras and Buffalo, 2010). Additionally, the evidence suggests that theta and gamma oscillations interact in order to encode and organize the working memory representations. Extrapolating this concept, it is proposed that this interaction might serve as a working memory buffer for the encoding of episodic memories (Klimesch et al., 2008; Nyhus and Curran, 2010).

However, this hypothesis still waits for conclusive empirical evidence because the areas that showed increased theta and gamma oscillations do not overlap perfectly (Sederberg et al., 2003, 2007; Osipova et al., 2006). Furthermore, to our knowledge, cross-frequency correlation, coherence or another measure of functional connectivity that involve both theta and gamma oscillations have not been explored during the encoding of episodic memory.

Theta and high gamma oscillations were higher during the encoding of Phase 2 stimuli in the emotional context and some areas of the left hemisphere showed increases in both frequency bands at similar time windows. Although functional connectivity was not tested, this partial overlapping suggests the interaction between theta and gamma activities.

We suggest that the stimuli processing within an emotional context is facilitated by mechanisms of expectancy, attention, and sensory processing that are recruited by the context itself, probably through a top–down control. We conclude that similar mechanisms are responsible for the subsequent memory effect for stimuli with emotional content or context. Despite each measure showed a unique pattern of activity related to the functional specialization of the cortical areas, N1, theta and gamma activity overlapped at the left prefrontal, frontal, parietal, and temporal locations. This lateralization is in line with the immense majority of the evidence that link the episodic memory encoding with the left hemisphere.

Taken together, this work does an important contribution to the characterization of the brain dynamics during the encoding of information in the emotional memory task and, by extension, adds new evidence about the brain processing of relevant environmental stimuli.

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Task-dependent and independent synchronous activity of monkey hippocampal neurons in real and virtual translocation

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Previous neurophysiological and behavioral studies relate hippocampal functions to place learning and memory, and encoding of task (or context)-specific information. Encoding of both task-specific information and own location is essential for episodic memory and for animals to navigate to reward-related places. It is suggested that different neural circuits with different assemblies of different hippocampal neurons are created in different environments or behavioral contexts for the hippocampal formation (HF) to encode and retrieve episodic memory. To investigate whether synchronous activity of hippocampal neurons, suggesting functional connectivity between those neurons, is task and position dependent, multiple single unit activities were recorded during performance of real and virtual translocation (VT) tasks. The monkey moved to one of four reward areas by driving a cab (real translocation) or by moving a pointer on a monitor. Of 163 neuron pairs, significant peaks in cross-correlograms (CCGs) were observed in 98 pairs. Most CCGs had positive peaks within 50 ms. Task-dependent cross-correlations (CCRs) were observed in 44% of the neuron pairs, and similarly observed in both the real and VT tasks. These CCRs were frequently observed in pyramidal vs. pyramidal neuron pairs with positive peak and peak shift. However, no consistent patterns of peak polarity, peak shift, and neuronal types were seen in task-independent CCRs. There was no significant difference in frequency of CCG peaks between real and VT tasks. These results suggest that the task-dependent information may be encoded by interaction among pyramidal neurons, and the common information across tasks may be encoded by interaction among pyramidal neurons and interneurons in the HF. These neuronal populations could provide a neural basis for episodic memory to disambiguously guide animals to places associated with reward in different situations.

Keywords: place cell, cross-correlation, auto-correlation, reference frame, episodic memory

INTRODUCTION

It has been suggested that food-storing birds use memory to retrieve stored food and have a larger hippocampal formation (HF; Clayton, 1995). The HF is important for encoding the association of taste with context (Gallo, 2005; Ho et al., 2011). The HF also plays an important role in a place-reward association in monkeys (Hampton et al., 2004), and monkey HF neurons differentially responded in a place-reward association task (Rolls and Xiang, 2005). Consistently, the HF is involved in conditioned place preference in which animals learned to prefer a chamber where a conditioned stimulus was associated with sucrose solution (Ito et al., 2006, 2008). These results suggest that the HF is important in mapping the location of rewards, and in guiding animals to places previously associated with reward.

Hippocampal formation neurons are active when the animal occupies a specific location (O'Keefe and Dostrovsky, 1971; McNaughton et al., 1983; Eichenbaum et al., 1987; Muller and Kubie, 1987). Unit-recording studies demonstrate that primate HF and parahippocampal gyrus (PH) neurons respond to spatial cues (O'Mara et al., 1994; Eifuku et al., 1995; Suzuki et al., 1997). Recent neuropsychological studies in humans have demonstrated a pivotal role of the medial temporal lobe including the HF and PH and its related areas in allocentric spatial information processing (Maguire et al., 1996a; Abrahams et al., 1997; Takahashi et al., 1997). Consistent with these studies, positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) have revealed blood flow increase in the HF and PH during various types of spatial tasks using a real or realistic virtual environment in normal humans (Aguirre et al., 1996; Ghaem et al., 1997; Maguire et al., 1997; Mellet et al., 2000).

It has been reported that activity of HF neurons is affected by the contexts of the tasks (Nishijo et al., 1993; Skaggs and McNaughton, 1998; Matsumura et al., 1999; Wood et al., 2000). This suggests that the HF encodes different reference frames; this might constitute a neural basis of episodic memory (Nishijo et al., 1993; Redish and Touretzky, 1997; Samsonovich and McNaughton, 1997; Skaggs and McNaughton, 1998). To understand the relationship between spatial selectivity and task-dependency of HF neurons, we have investigated the neuronal activities in allocentric space in real and virtual translocation (VT), in which the monkey in a movable cab could freely move toward a destination by manipulating a joystick (Ono et al., 1993; Nishijo et al., 1997; Matsumura et al., 1999; Hori et al., 2003). The results indicated that activity of 40% of monkey HF neurons was

location-selective, and that most neurons showed task-dependent responses. This suggests that the HF is crucial in allocentric information processing, and that the HF may encode contexts of reference frames. Furthermore, the HF neurons display different spatial responses depending on motivational states (hunger or thirst) and reward (food or water; Kennedy and Shapiro, 2009), and on movement trajectories (Frank et al., 2000; Wood et al., 2000; Dayawansa et al., 2006). These results suggest that the HF forms multiple representations of the outer world depending on these factors. Previous theoretical studies proposed that the HF represented the external world by a reference frame (Redish and Touretzky, 1997) or chart (Samsonovich and McNaughton, 1997) system in which different neural circuits with different assemblies of different HF neurons were created in different environments or behavioral contexts.

To investigate neuronal ensemble activity with functional connectivity, cross-correlation (CCR) has been applied in studies of HF neurons using awake rats (Sakurai, 1996; Tabuchi et al., 2000; Hirase et al., 2001; Takahashi and Sakurai, 2009). Activity patterns of neuronal ensembles and degree of temporal correlation in CCRs were correlated to behavioral performance or behavioral states in the rodent striatum (Jog et al., 1999) and HF (Hirase et al., 2001). We hypothesized that the neuronal networks in the HF encode contexts of reference frames, by which appropriate behaviors in given situations might be chosen, i.e., different neural circuits in the HF might encode respective contexts. In the present study, to determine whether the functional neuronal connectivity among the HF neurons changes across different tasks, we reanalyzed the data set of the monkey HF neuronal activity during performance of the four different real and VT tasks (Matsumura et al., 1999) using a shift register difference CCR analysis.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL APPARATUS

Two adult monkeys (*Macaca fuscata*), weighing 4.2 and 5.6 kg, were used. The experimental protocol was described in detail in the previous paper (Matsumura et al., 1999). The monkeys were treated in strict compliance with the United States Public Health Service Policy on Human Care and Use of Laboratory Animals and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the Guideline for the Care and Use of Laboratory Animals in our university (University of Toyama), and all efforts were made to minimize the number of animals used and their suffering.

The monkey was restrained painlessly in a stereotaxic apparatus by a surgically fixed head holder and sat in a 0.7 m × 0.8 m × 0.85 m high cab, which could freely move in a 2.5 m × 2.5 m experimental field located in a 5.0 m × 6.0 m room. The front wall of the cab was made of transparent Plexiglass and the rear wall consisted of a steel plate with two speakers. In the upper part of the front wall there was a color liquid crystal display (LCD) monitor (diagonal, 26 cm) with a resolution of 640 × 480 pixels (LMD-1040XC, Sony), 25 cm from the monkey, on which visual stimuli were displayed. The lower part of the front wall contained a joystick used to move the cab in the experimental field and/or a pointer (an yellow circle with a radius of 3 mm) on the LCD monitor. The cab and/or the pointer could be moved at a constant velocity (cab, 3 cm/s in the experimental field; pointer, 2.5 mm/s on the LCD monitor) in all four directions during the continuous manipulation of the joystick by the monkey.

The room, illuminated at 140 lux, contained several readily identified landmarks (sink, stereomicroscope, refrigerator, etc.; **Figure 1Aa**). The monkey sat in a chair within the cab and could see these visual landmarks in the experimental room, which were available to identify its position in the experimental field. The monkey performed four behavioral tasks under the guidance of visual stimuli on the LCD monitor and auditory stimuli from the two speakers. The tasks was to move the cab to one of four reward areas (target areas with a radius of 20 cm) in the experimental field [real translocation (RT) task], or to move the pointer to one of four reward areas (target circles with a radius of 16 mm) on the LCD monitor VT task by manipulating the joystick. The two-dimensional space on the LCD monitor was proportional to the experimental field (1:12.61; **Figure 1Ab**).

BEHAVIORAL PARADIGMS

There were four kinds of behavioral tasks based on a combination of cab movements, indication of the pointer, and a target circle on the LCD monitor as described below. Time charts of the four behavioral tasks are shown in **Figure 1B**. More detailed methods were described previously (Matsumura et al., 1999).

REAL TRANSLOCATION TASK WITHOUT A POINTER UNDER CONTINUOUS PRESENTATION OF A TARGET CIRCLE ON THE LCD MONITOR (RT/TC TASK)

After the cab was placed at a starting point, the task was initiated by a 1-s presentation of a warning tone (1300 Hz), followed by presentation of a 20 cm × 20 cm blue square frame on the LCD monitor, which corresponded to the 2.5 m × 2.5 m experimental field (warning phase). In this task, the blue square frame worked as a kind of a map in the experimental field in a scale ratio of 1:12.61 (**Figure 1Ab**). Then, a target circle (a red circle with a radius of 1.6 cm) appeared at one of the four corners of the blue frame. Respective target areas in the experimental field (i.e., destination of the cab) corresponded to the four target circles on the LCD monitor. After 2 s presentation of the target circle (discrimination phase), the monkey manipulated the joystick to drive the cab toward the target area by recognizing its own location from the landmarks in the experimental field (manipulating/response phase). When the monkey arrived at the target area, a reward of approximately 6 ml of orange juice was given for 3 s (reward phase). After delivery of a juice reward, the warning tone was stopped and the blue square frame and target circle disappeared. If the monkey failed to drive the cab to the target area within 120 s, the trial was terminated and reward was withheld. At the end of each trial the cab was moved to the next starting point under a computer control. Time interval between trials was 30–60 s.

REAL TRANSLOCATION TASK WITH A POINTER UNDER CONTINUOUS PRESENTATION OF A TARGET CIRCLE ON THE LCD MONITOR (RT/P-TC TASK)

The task sequence and behavioral requirements were similar to those in the RT/TC task except that the pointer, indicating the location of the cab, was shown on the LCD monitor. Here, the monkey

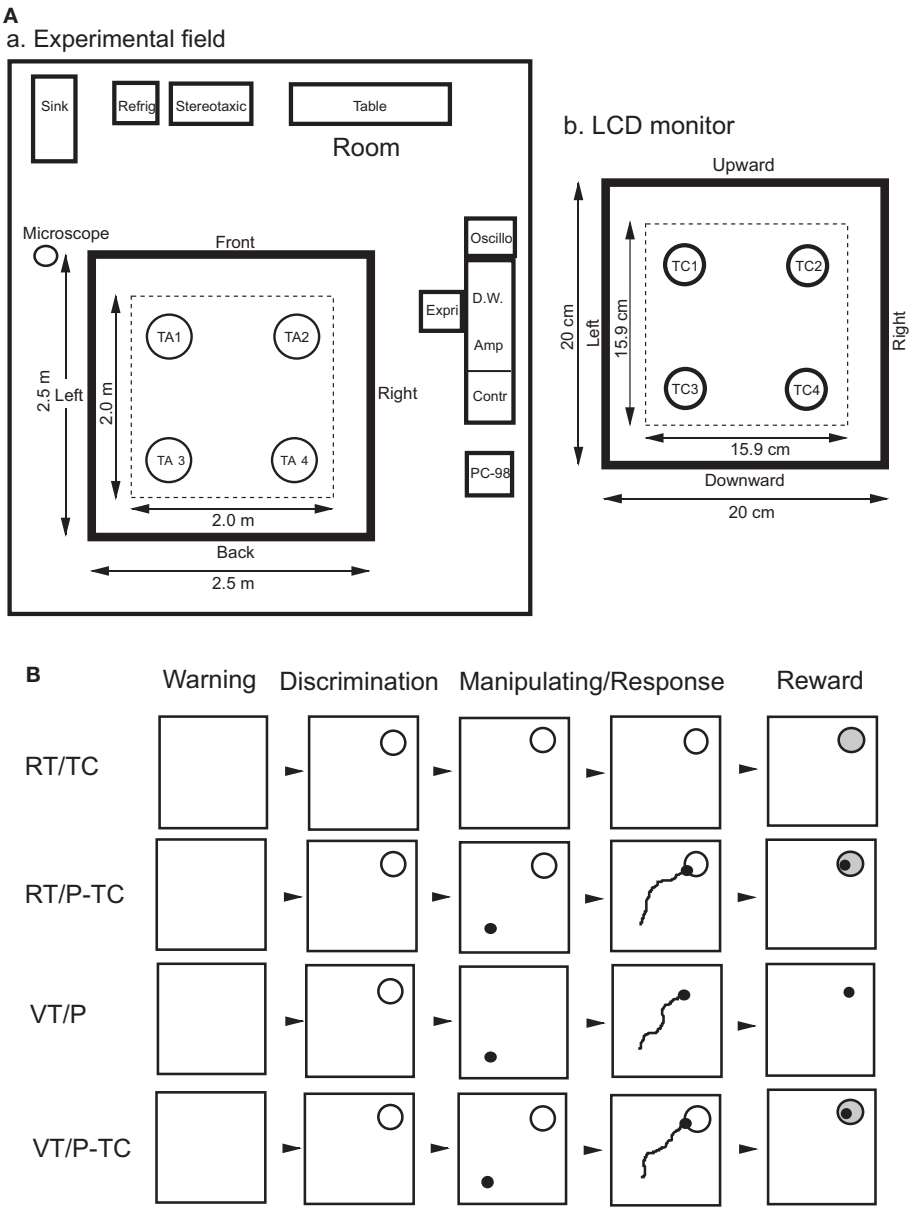

FIGURE 1 | Experimental set up (A) and task paradigms (B). (A) Spatial arrangements of room cues and target areas for the real translocation (RT) task (a) and those of target circles on the LCD monitor for the RT and virtual translocation (VT) tasks (b). In the RT tasks (a), the monkey moved the cab to one of four reward areas in the four corners of the experimental field [target area (TA) 1, 2, 3, or 4] by manipulating the joystick. Area in a thick line square, 2.5 m × 2.5 m experimental field; area in a dotted line square, range of movement of a center of the cab where the monkey sat in a chair. *Refrig*, refrigerator; *Stereotaxic*, stereotaxic apparatus; *Oscillo*, oscilloscope; *Expri*, experimenter; D.W., microcomputer for Datawave; *Amp*, main amplifier; *Contr*, task controller; PC-98, microcomputer for monitoring movements of the cab and joystick. In the VT tasks (b) the monkey moved a pointer on the LCD monitor to one of four reward areas in the four corners of the LCD monitor [target circle (TC) 1, 2, 3, or 4] by manipulating the joystick. Area in a dotted line square, range of movement of the pointer on the LCD monitor. **(B)** Time chart of the four behavioral tasks. Each square indicates the frame which appeared on the color liquid crystal display (LCD) monitor. All four tasks were consisted of four phases; (1) warning phase: the warning tone and the frame were presented for 1 s, (2) discrimination phase: a red target circle (radius, 15.9 mm) appeared on the LCD monitor for 2 s, (3) manipulating/response phase: a monkey manipulated a joystick to drive a cab or move a pointer on the LCD monitor, and (4) reward phase: approximate 6 ml of orange juice was delivered for 3 s. In the real translocation (RT) tasks, one of four target circles (TC), corresponding to one of four target areas in the experimental field, was presented on the LCD monitor. Then the monkey manipulated the joystick to drive the cab toward the indicated target area with (RT/P-TC task) or without (RT/TC task) guidance of a pointer (P), indicating location of the cab, on the monitor. In the virtual translocation (VT) tasks, the cab was located stationally throughout the trial. After one of the target circles was presented on the LCD monitor, the monkey manipulated the joystick to move a pointer toward the indicated location of the target circle without (VT/P task) or with (VT/P-TC task) presentation of the target circle during the manipulating/response phase.

could use the LCD monitor as a map of the experimental field on which location of the monkey was indicated by the pointer. This is a similar situation to that for a car navigator, in which location of a car is indicated in a map on a LCD display. The task phases were same as those of the RT/TC task.

VIRTUAL TRANSLOCATION TASK WITH A POINTER (VT/P TASK)

The cab was stationary throughout the trial. A target circle was transiently presented on the LCD monitor only during the discrimination phase. Therefore, the monkey was required to memorize the location of the target circle during the discrimination phase, and to move the pointer to the location based on memory by manipulating the joystick. The task phases were same as those in the RT/TC task.

VIRTUAL TRANSLOCATION TASK WITH A POINTER UNDER CONTINUOUS PRESENTATION OF A TARGET CIRCLE ON THE LCD MONITOR (VT/P-TC TASK)

The task sequence and behavioral requirements were similar to those in the VT/P task except that a target circle was presented during the discrimination and manipulating/response phases. The task phases were same as those in the RT/TC task.

CHARACTERISTICS OF EACH TASK

These four tasks were so designed for behavioral requirement (i.e., joystick manipulation) and reward (juice) as to be similar across the tasks. However, different knowledge systems were assumed to be required to accomplish each task. It is noted that the pointer in the LCD monitor was not presented in the RT/TC task. In addition, the monkey always faced in the fixed direction, and consequently the same landmarks were seen in each trial. Therefore, the monkey had to judge its position based on the survey knowledge of the landmarks (Thorndyke and Hayes-Roth, 1982; Aguirre and D'Esposito, 1997) in the experimental room in the RT/TC task (i.e., relative spatial knowledge of place). Furthermore, the monkey was able to change its course flexibly during translocation when movement direction deviated from the destination (Matsumura et al., 1999). These support an idea that the monkey's behavior was based on a cognitive map in the brain (locale system), in which the spatial relationships of various landmarks are represented, rather than taxon systems where a set of stimulus–response (e.g., single landmarks to actions or movements) associations are represented (O'Keefe and Nadel, 1978). The situation in the RT/TC task corresponds to those in the previous human studies in which human subjects with temporal lobe lesions displayed deficits in topographical orientation such as way-finding from environmental video films (Maguire et al., 1996a), and in which cerebral blood flow in the HF of normal human subjects increased during learning of navigation routes from video films (Maguire et al., 1996b). In contrast, the monkey did not necessarily judge its position based on the cognitive maps in the RT/P-TC task since its position was indicated by the pointer on the LCD monitor. It is noted that behavioral requirements in the RT/P-TC task were similar to those in the RT/TC tasks except this point.

In the VT tasks cognitive spatial information processing (information processing in the locale system) was not necessary since the pointer and/or a target circle were continuously presented on the monitor and the cab was located in a fixed place. The VT/P task required the monkey to memorize location of the target area on the monitor, so that this task was employed to test two-dimensional spatial short-term memory. Since locations of both the target area and the monkey or pointer were indicated in the RT/P-TC and VT/P-TC tasks, the situations in these two tasks correspond to a visible platform condition in a water maze paradigm for rodents in which animals with HF lesions performed normally (Morris et al., 1982). In spite of these differences among the four tasks, all these four tasks required to some extent spatial information processing.

TRAINING AND SURGERY

The monkey was initially trained to learn the VT/P-TC task. However, the monkey was required to move the pointer on the LCD monitor in fixed directions (i.e., forward–backward, leftward–rightward, or diagonally) by manipulating a joystick that had physical limitations of movements. It took about 2–3 months for the monkey to learn moving the pointer freely in all directions using a joystick without physical limitations. When the monkey learned to perform the VT/P-TC task with a criterion of 95% correct responses, it was then trained on the VT/P task. The monkey required about 3 months of training to reach 95% performance in the VT/P task. In the next stage, the monkey was trained to learn the RT/P-TC task. It learned the task very easily. After learning these three tasks with 95% performance, the monkey was trained in the RT/TC task. It took about 5 months of training for it to reach 95% performance. In the final stage, the monkey was well trained and performed all tasks for 3 weeks. The monkey was trained for 3 h/day and 5 day/week.

After completion of this training period, a head-restraining device (a U-shaped aluminum plate) was attached to the skull under aseptic condition and sodium pentobarbital anesthesia (35 mg/kg, i.m.). The plate was anchored with dental acrylic to stainless steel bolts inserted in keyhole slots in the skull. During the surgery, heart and respiratory functions and rectal temperature were monitored on a polygraph system (Nihon Kohden, Tokyo, Japan). The rectal temperature was controlled at 37 ± 0.5°C by a blanket heater. Antibiotics were administrated topically and systemically for 1 week to protect against infection. Two weeks after surgery, the monkey was retrained. Performance criterion was again attained in about 10 days. All experimental protocols were performed in accordance with the guidelines for care and use of laboratory animals approved by the University of Toyama and the National Institutes of Health's Guide for the Care and Use of Laboratory Animals, and approved by the Committee for Animal Experiments at the Toyama Medical and Pharmaceutical University (University of Toyama).

RECORDING PROCEDURES AND DATA ACQUISITION

After recovery from training and surgery, extracellular unit activities were recorded from the HF and PH using a tungsten electrode while the head of the monkeys were painlessly fixed in the stereotaxic apparatus using the surgically implanted U-shaped aluminum plate (see Matsumura et al., 1999). A glass-insulated tungsten microelectrode (1–2 MΩ at 1000 Hz) was stereotaxically inserted vertically into the HF and PH stepwise by a pulse motor-driven manipulator (SM-21; Narishige, Tokyo, Japan). The diameter of the tungsten electrodes was less than 5 μm. The mean firing rates of the HF and PH putative pyramidal neurons (place

cells) were less than 1 spikes/s (Matsumura et al., 1999), which are comparable to those in the monkey HF by Skaggs et al. (2007) using tetrodes. Outputs from the amplifier were digitized and sent to an IBM-compatible 486-based microcomputer. The software (Enhanced Discovery, DataWave Technologies) collected an epoch of the digitized analog signals for every event which exceeded a user-set threshold. The digital outputs of X and Y coordinates of the cab and/or pointer were simultaneously displayed on-line on another monitor (PC-9821, NEC). Each task consisted of 12 trials which contained 12 different combinations of starting points and target areas (4 starting points × 3 target areas). Eye movements were also monitored by an eye monitor system using an infrared charge-coupled device (CCD) camera (EM100, Toyo Sangyo Co., Ltd.).

SINGLE UNIT ISOLATION

One to four single units were isolated by means of off-line cluster analysis from a single channel. Single unit isolation was performed manually using eight waveform parameters (positive, negative, and entire spike amplitudes, spike duration, amplitude windows immediately prior to and after the initial negative-going peak, and time until maximum of positive and negative peaks) with the DataWave software. First, units were isolated using parameters of entire spike amplitude and spike duration. Then, clusters of the isolated units were further analyzed using other pairs of these eight parameters. Finally, superimposed waveforms of the isolated units were drawn to check invariability of the waveforms. Invariability of the waveforms was also checked during the recording sessions; data were discarded if they were not identical in early and late recording period. Superimposed waveforms were checked so that no overcut and overlap was performed for the single unit isolation. We further examined autocorrelograms (ACRs; bin, 1 ms; bin width, 50 ms) for neurons to verify that the refractory period was greater than 1–2 ms.

CLASSIFICATION OF ISOLATED UNITS

There have been no reports indicating electrophysiological characteristics of pyramidal neurons and interneurons in the HF of awake monkeys. Therefore, the isolated units were classified based on the rodent criteria: pyramidal neurons and interneurons were identified based on their firing rates and spike widths (Fox and Ranck, 1981; Mizumori et al., 1990; Jung et al., 1994). The data matrix of the firing rates and spike widths was analyzed by the cluster analysis (SPSS version 10.0.7J, SPSS Inc.,) utilizing Pearson's product-moment correlation coefficients and the average linkage method (Bieber and Smith, 1986; Nishijo and Norgren, 1990, 1991). The results indicated four clusters, and these four clusters were further grouped into two clusters, i.e., pyramidal neurons vs. interneurons based on neurophysiological characteristics (Csicsvari et al., 1998, 1999; Wiebe and Stäubli, 2001; see **Figure 2** in the results). The mean firing rate of each isolated single unit (spike) was computed from whole data across the four tasks (12 trials × 4 tasks). The duration of spikes were measured at 25% of maximum spike amplitude (Csicsvari et al., 1999).

FIRING RATE MAPS AND PLACE FIELDS

In order to visualize firing patterns with respect to location of the monkey in the experimental field or the pointer on the LCD monitor, firing rate maps were created (Matsumura et al., 1999). Movable

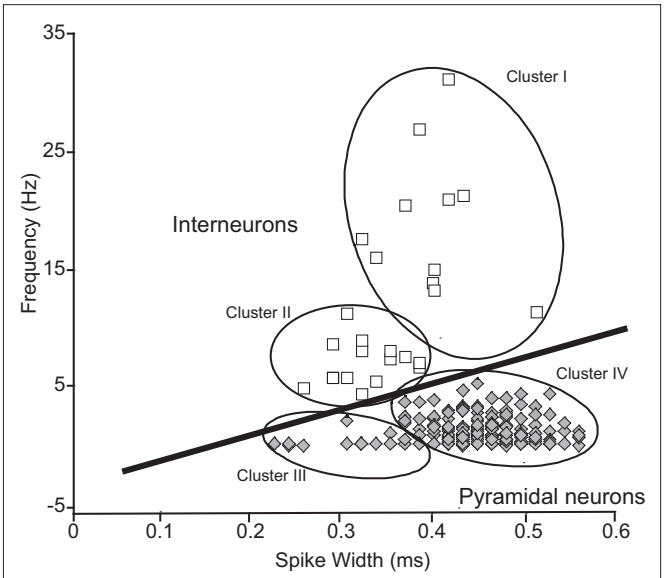

FIGURE 2 | Classification of HF neurons on the basis of physiological parameters in extracellular neuronal recordings. A linear cut-off method in firing rate–spike width space (Wiebe and Stäubli, 2001) to classify of pyramidal neurons and interneurons. HF neurons were initially grouped into the four clusters (I–IV) according to the cluster analysis. These four clusters were further grouped into two clusters (pyramidal neurons and interneurons) by the linear cut-off method. The population of pyramidal neurons was statistically well separated in the two-dimensional space [$F(2,417) = 357$, $P < 0.0001$ by two-way ANOVA]. Note that the distribution pattern and characteristics of waveforms of the pyramidal neuron and interneurons are comparable to those in rats (Jung et al., 1994; Csicsvari et al., 1999; Wiebe and Stäubli, 2001).

area in the experimental field (2.0 m × 2.0 m) and that on the LCD monitor (15.9 cm × 15.9 cm) were divided into 64 pixels by an 8 × 8 array. The mean firing rate for each pixel was calculated as the average spikes per s for all visits to that pixel during the manipulating/response and reward phases. Then, a grand mean firing rate (M) was calculated by averaging the mean firing rate across all pixels. Neuronal activity in each pixel was expressed as a relative firing rate (R) in which the mean firing rate in each pixel was divided by the grand mean in each task, and shown in five steps ($R \geq 2M$; $2.0M > R \geq 1.5M$; $1.5M > R \geq 1.0M$; $1.0M > R \geq 0.5M$; $R < 0.5M$).

To determine the boundary of the place field of the HF and PH neurons where the activity increased, we compared firing rate of each pixel with a mean firing rate over a given task (Matsumura et al., 1999). An increase in firing rates in each pixel was defined as that greater than 2.0 times the mean firing rate for a given neuron in each task. This criterion was similar to those in the previous studies (Muller et al., 1987; Kobayashi et al., 1997). Clusters of the pixels with firing rates exceeding both 1.5 and 2.0 times of the mean firing rate were identified. All pixels that did not satisfy this criterion were eliminated. Then, only place fields that had at least 1 pixel with a firing rate exceeding 2.0 times the mean and 1 adjacent pixel with a firing rate exceeding 1.5 times the mean were identified. Finally, the place fields, within which all firing rates of the pixels were less than 2.0 spikes/s (Muller et al., 1987; Breese et al., 1989), were excluded. The remained place fields could be expanded through any edge shared by 2 pixels meeting the criterion (greater

than 1.5 times the grand mean). If one or more neighboring pixels satisfied the criterion, the field was expanded to include the pixel(s). Each added pixel was then tested for the presence of a neighboring pixel that met the criterion. When no neighboring pixel satisfied the criterion, the limit of the field was identified. Boundaries of a place field were established by constructing a rectangle that had one diagonal connecting the minimum X and Y coordinates with the maximum X and Y coordinates.

CCR AND ACR ANALYSIS

Shift register difference CCRs with a 5 ms bin width and a ±300 ms binning range were employed over 12 trials in each task. Each observation time comprised about 150 s period including a trial and an inter-trial interval. To make a shift register difference CCR, a raw CCR was first calculated by counting the discharge numbers of target cells at every discharge of reference cells in each 5 ms bin over ±300 ms. Second, a shift register CCR was computed between discharges of a reference cell synchronized with an event trigger (start trigger of each trial) and discharges of a target cell synchronized with a subsequent event trigger. Then, the latter CCR was subtracted from the former CCR, which could result in negative peaks. This shift register difference CCR can prevent detection of pseudo positive peaks when two cells have a same stimulus dependence and behavioral correlate (Aertsen et al., 1989; Munk et al., 1995; Nowak et al., 1995). Finally, spike counts in each bin of the CCR histograms were normalized by division of a sum of the reference and target cell discharges according to the method by Hirase et al. (2001). The criterion for statistical significance was considered to be $P < 0.001$ of confidence limits according to the methods by Perkel et al. (1967) and Gochin et al. (1989). Confidence limits are based on the assumption that, for independently firing spike trains, counts in every bins of cross-correlogram (CCG) are modeled as a Poisson process (Perkel et al., 1967; Gochin et al., 1989). DataWave system proprietary programs were used for CCR analyses.

Additional CCR analyses were further performed with a 1 ms bin width and a ±30 ms binning range when the time difference of significant highest peaks were close to 0 ms, to analyze a precise distribution of the highest peaks (see examples in **Figures 4Ad and 5Ab**). Data of time differences from −1 to +1 ms was discarded due to the limit of temporal precision from a single electrode.

Task-dependency of CCRs is defined as such when shift register difference CCRs showed significance in at least one but not all four tasks. We also defined that neuronal pairs are without peak shift, when their CCG peaks were centered at 0 ms (CCG peaks were generally symmetrical). In some neuron pairs with symmetrical CCG peaks centered at about time zero, their peak points could not be discerned precisely because of broad peaks. They were included in a group without a peak shift. Also, discharge patterns of each of neuron pairs were analyzed with ACRs. Spike counts in each bin of the ACR histograms were normalized by division of a sum of the discharges of the neuron.

RESULTS

IDENTIFICATION OF PYRAMIDAL NEURONS AND INTERNEURONS BY EXTRACELLULAR RECORDINGS

Distributions of the monkey HF neurons in firing rate–spike width space are shown in **Figure 2**. The cluster analysis indicated four clusters of the HF neurons (Clusters I–IV). The previous results in rodents reported that typical interneurons had relatively narrow duration and high firing rates (>5 Hz) while pyramidal neurons had wide duration and low firing rates (<5 Hz; Csicsvari et al., 1998, 1999; Wiebe and Stäubli, 2001). Based on these results, Clusters I and II, and Clusters III and IV were combined to form two new larger clusters (open squares vs. shaded diamonds). This distribution pattern was well comparable to that in rodents (Csicsvari et al., 1998, 1999; Wiebe and Stäubli, 2001); Clusters I and II, and Clusters III and IV corresponded interneurons and pyramidal neurons, respectively. Typical waveforms of pyramidal neurons and interneurons are shown in **Figure 3**, in which the two neuron pairs from A to F were recorded from the same electrodes.

CHARACTERISTICS OF HF NEURONS AND TYPES OF NEURON PAIRS

The 209 HF and PH neurons (pyramidal neurons, 183; interneurons, 26) which had more than one isolated neuron partner recorded simultaneously were isolated. Of these 209 neurons, 163 neuron pairs were identified. The mean firing rate of the putative pyramidal neurons was 1.25 ± 1.18 spikes/s (mean ± SD; range: 0.01–5.20) and those of the putative interneurons was 12.39 ± 7.67 spikes/s (range: 5.45–31.09). The number of neuron pairs in each subregion of the HF and PH, classified by types of neuron pairs, is shown in **Table 1**. Of the 163 neuron pairs, 98 showed significant peaks in the CCR histograms. Thus, about two thirds of the neuron pairs had significant peaks in at least one CCR histogram of the four tasks. The most common type of the neuron pairs consisted of two pyramidal neurons (118 pairs), followed by pyramidal neuron and interneuron (32 pairs). The least common type was that of two interneurons (13 pairs). These results were attributed to the fact that the number of pyramidal neurons was larger than that of interneurons (about 10% of pyramidal neurons) in the HF (Woodson et al., 1989; Aika et al., 1994).

TASK-DEPENDENT CCRS WITH OR WITHOUT CHANGES OF THE FIRING RATES

An example of a task-dependent CCG peak for a pair of CA1 pyramidal neurons (529U1 and 529U2, these waveforms are shown in **Figures 3Aa,b**, respectively) is shown in **Figure 4A**. This pair showed significantly higher CCG peak only in the VT/P-TC task, indicating that the functional connectivity between these two neurons strengthened when spatial information on the LCD monitor was required. The CCR histogram with 1 ms bin width (inset in **Figure 4Ad**) indicated that the highest peak shifted 3 ms from time zero. It should be noted that this positive peak was independent of the average firing rates during the overall recording session in each task since spike counts in each bin of the CCR histograms were normalized by dividing by the sum of the both reference and target cell discharges. ACRs of these neurons showed sharp peaks in 529U1 (**Figure 4B**) and broad peaks in 529U2 (**Figure 4C**). These ACRs indicate that 529U1 discharged sporadically in a bursting manner, and 529U2 discharged relatively tonically. This bursting pattern of the both neurons became more evident in the VT/P-TC task (**Figures 4Bd,Cd**), in which significant CCR was observed. Weak 10–14 Hz oscillatory activity was also observed in neuron 529U1.

Figure 5A shows another example of a task-dependent CCG peak, independent of the average firing rate, for a pair of CA1 pyramidal neurons (728U1 and 728U2, these waveforms are

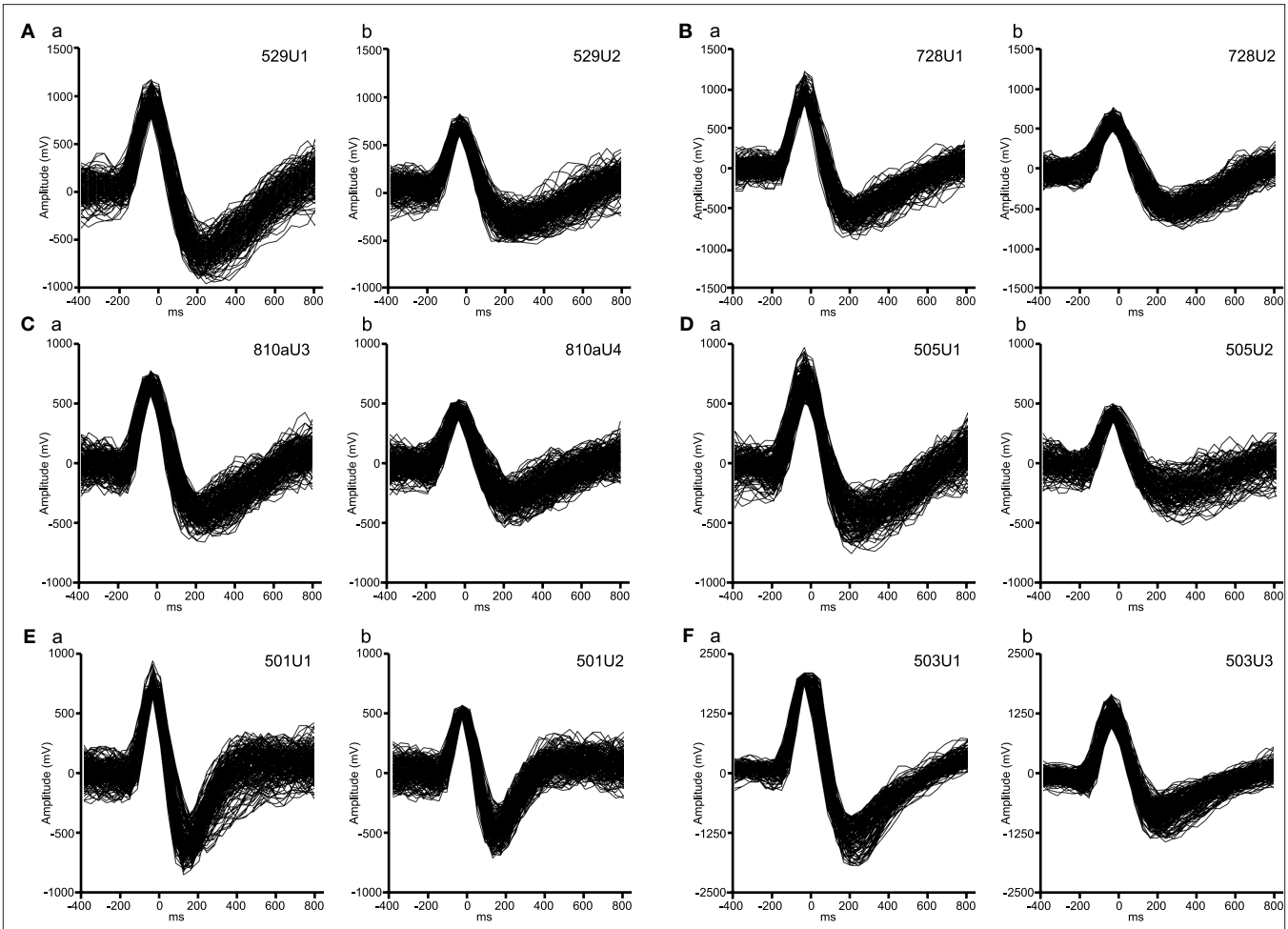

FIGURE 3 | Typical waveforms of putative pyramidal neurons (Aa, Ab, Ba, Bb, Ca, Cb, Da, Ea, Fa, Fb) and an interneuron (Db, Eb) isolated by off-line cluster analysis. The two units in a same uppercase letters were simultaneously recorded from a same electrode. The ACR and CCR of these neurons were shown in **Figures 4–10**. The unit pairs of **(A)** (**Figure 4**) and **(B)** (**Figure 5**) show task-dependent CCRs, whereas the unit pairs of **(C)** (**Figure 6**), **(D)** (**Figure 7**), and **(E)** (**Figure 8**) show task-independent CCRs. The unit pair **(F)** (**Figure 10**) shows task-dependent CCG peak with trigger-like activity.

shown in **Figures 3Ba,b**, respectively). This pair showed a significant CCG peak only in the RT/P-TC task. This suggests that the functional connectivity strengthened when both real and virtual information was required. Similarly, this pair had a peak shift in 4 ms (inset in **Figure 5Ab**). ACRs of these neurons (**Figures 5B,C**) showed generally similar patterns to those of the neuron pair in **Figure 4**. Although 728U1 discharged in a similar bursting manner across the tasks, bursting pattern of 728U2 became more evident in the RT/P-TC task (**Figure 5Cb**) in which significant CCR was observed.

Thus, the neuron pairs with task-dependent CCG peaks had some common characteristics. First, most pairs were comprised of pyramidal vs. pyramidal neurons. Especially, when the significant CCG peaks were observed only in one task, all the pairs were pyramidal vs. pyramidal neurons. Second, almost all CCG peaks were positive. Especially, all pairs had positive peaks when the significant CCR was selective only to one task. Third, CCG peaks of most pairs shifted with time differences less than 50 ms (see also **Figure 13**).

TASK-INDEPENDENT CCRs

Figure 6A shows a typical example of positive peaks in CCR histograms (±300 ms binning range, 5 ms bin width), between a pair of CA1 pyramidal neurons (810aU3 and 810aU4), which occurred non-differentially in the four tasks. Significant positive peaks, which centered at about time zero with symmetrically broad bases between about ±200 ms, were observed in all four CCR histograms. These data indicated that the two neurons discharged mostly at same time, and synchrony of the two neurons was task-independent. This CCR pattern was frequently observed in the combinations of pyramidal vs. pyramidal neurons and of pyramidal neuron vs. interneuron. Their peaks occurred at about time zero (no clear peak shift) in half of the pairs of pyramidal neuron vs. interneuron, while most pairs of pyramidal vs. pyramidal neurons displayed a peak shift. ACRs (±300 ms binning range and 5 ms bin width) of these neurons are shown in **Figure 6B**. Both neurons 810aU3 (**Figure 6Ba**) and 810aU4 (**Figure 6Bb**) showed a bell-shaped ACR. This suggests that these neurons discharged in a similar bursting rhythm.

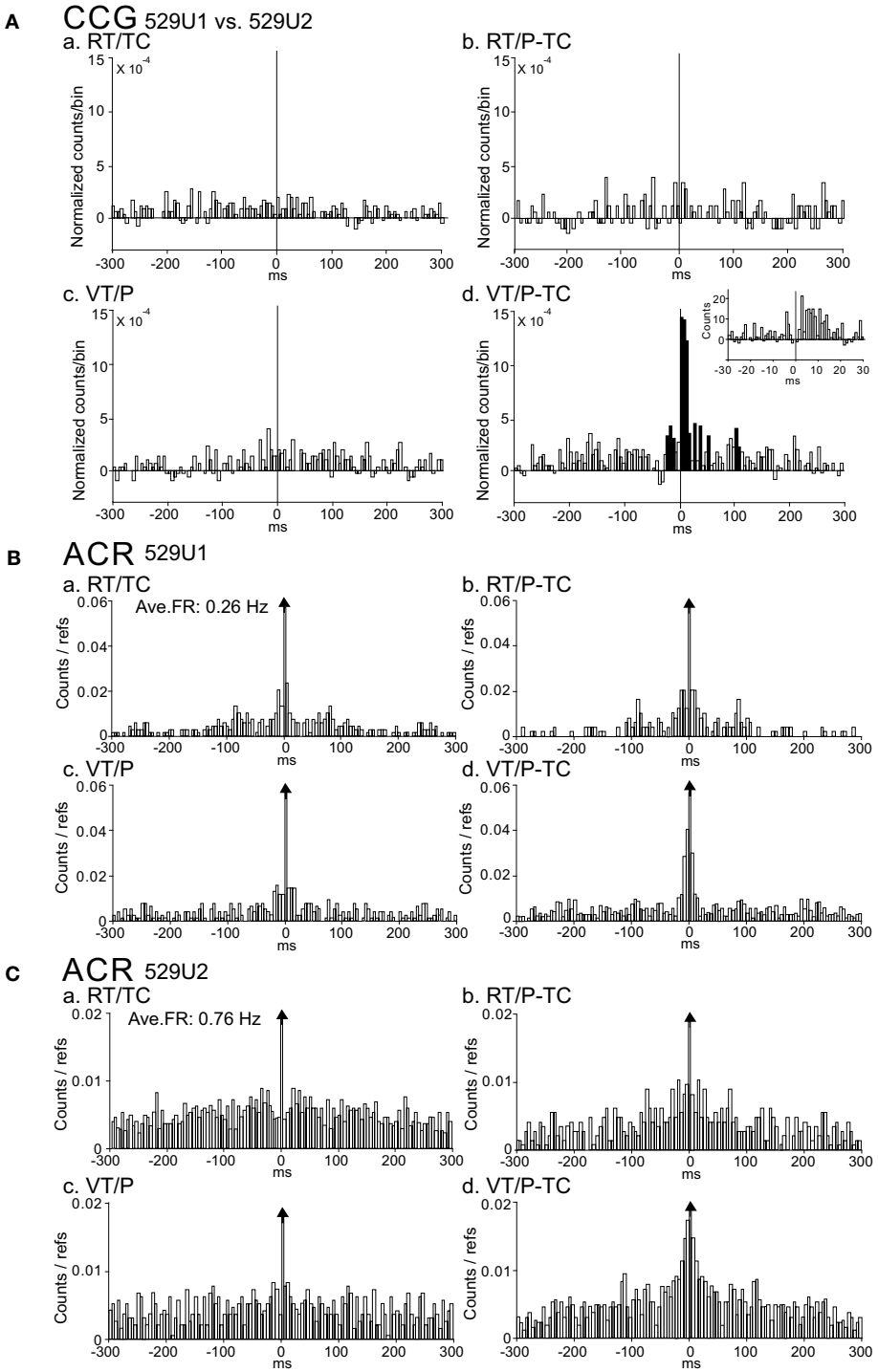

FIGURE 4 | An example of a neuron pair displaying a task-dependent peak in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neurons (529U1, reference cell; 529U2, target cell) in the four tasks **(a–d)**. Note a significant peak in the VT/TC-P task. **(d)**. Inset in **(d)**, CCR histogram with 1 ms bin width. Total spikes analyzed in each CCG in each task for neurons 529U1 and 529U2 were 382 and 1419 spikes in the RT/TC, 203 and 606 spikes in the RT/P-TC, 333 and 745 spikes in the VT/P, and 282 and 609 spikes in the VT/P-TC tasks, respectively. Hatched bins indicate those exceeding 99.9% of confidence limits. Ordinate, spike counts of the referenced cell (spike counts in each bin of the CCR histograms were divided by both sums of the reference and target cell discharges). Abscissa; time difference from each spike of the reference cell to that of the target cell. **(B,C)** ACRs of the same neurons in **(A)** [**(B)** 529U1; **(C)** 529U2]. Note a sharp peak in 529U1, and a broad peak in 529U2. It is noted that 529U1 and 529U2 were not complex spike cells. Ave. FR, average frequency of a neuron across the four tasks. Ordinate, spike counts of the cell. Abscissa; time difference from each reference spike to each spike of the cell.

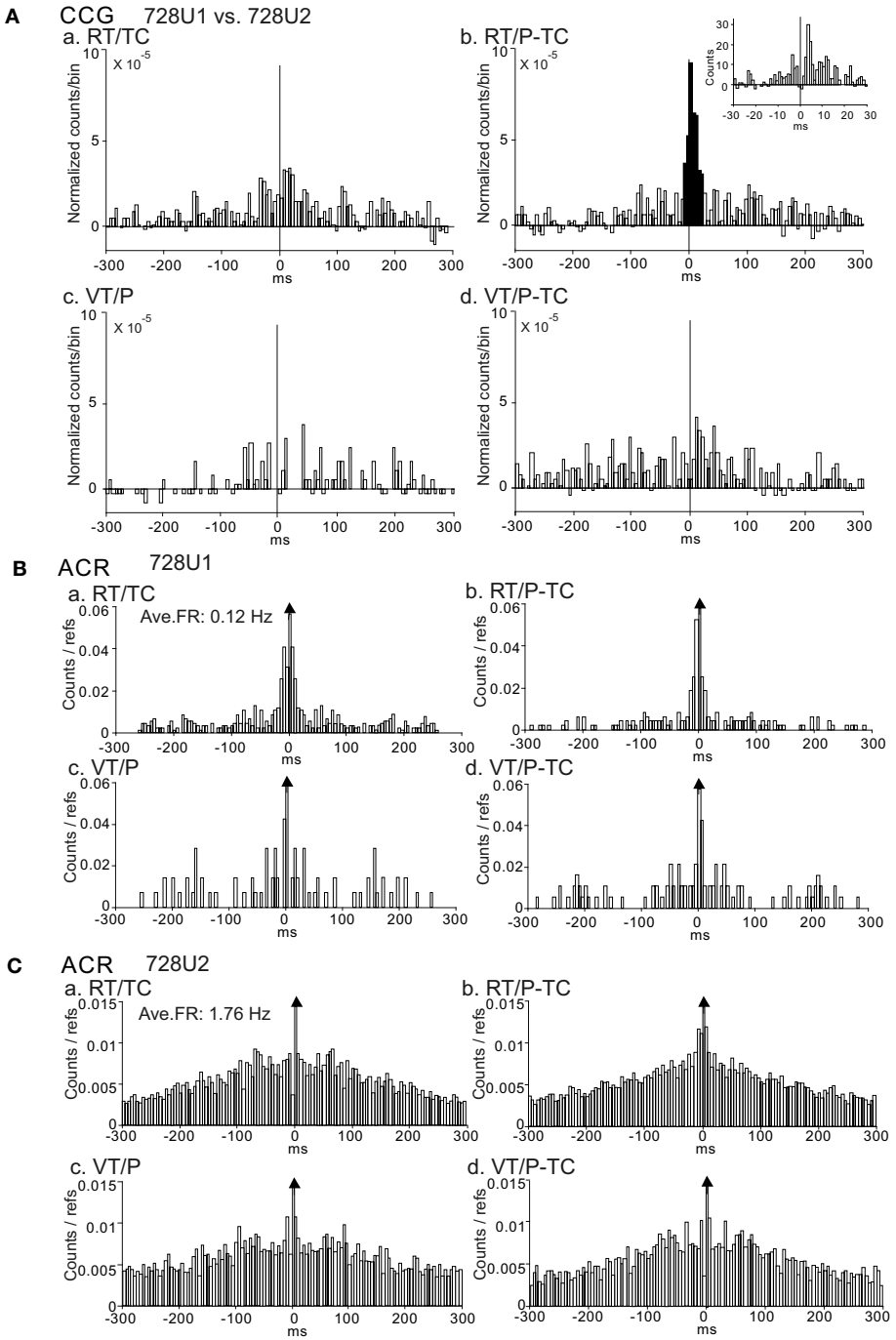

FIGURE 5 | An example of a neuron pair displaying a task-dependent peak in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neurons (728U1, reference cell; 728U2, target cell) in the four tasks **(a–d)**. Note a significant peak in the RT/P-TC task **(b)**. Inset in **(b)**, CCR histogram with 1 ms bin width. Total spikes analyzed in each CCG in each task for neurons 728U1 and 728U2 were 264 and 2006 spikes in the RT/TC, 340 and 3034 spikes in the RT/P-TC, 91 and 1966 spikes in the VT/P, and 163 and 1946 spikes in the VT/P-TC tasks, respectively. **(B,C)** ACRs of the same neurons in **(A)** [**(B)** 728U1; **(C)** 728U2]. Note a sharp peak in 728U1, and a broad peak in 728U2. It is noted that 728U1 and 728U2 were not complex spike cells. Other descriptions as for **Figure 4**.

A typical example of task-independent negative CCG peaks between a pair of CA1 pyramidal neuron (505U1: reference neuron) and interneuron (505U2: target neuron) is shown in **Figure 7**. Significant negative peaks centered at about time zero, with symmetrically broad bases ranging from −35 to +35 ms latency, were observed in all CCR histograms in the four tasks (**Figure 7A**). These results indicate that the synchrony of these neurons is task-independent. Patterns of ACRs in these neurons (505U1 and 505U2)

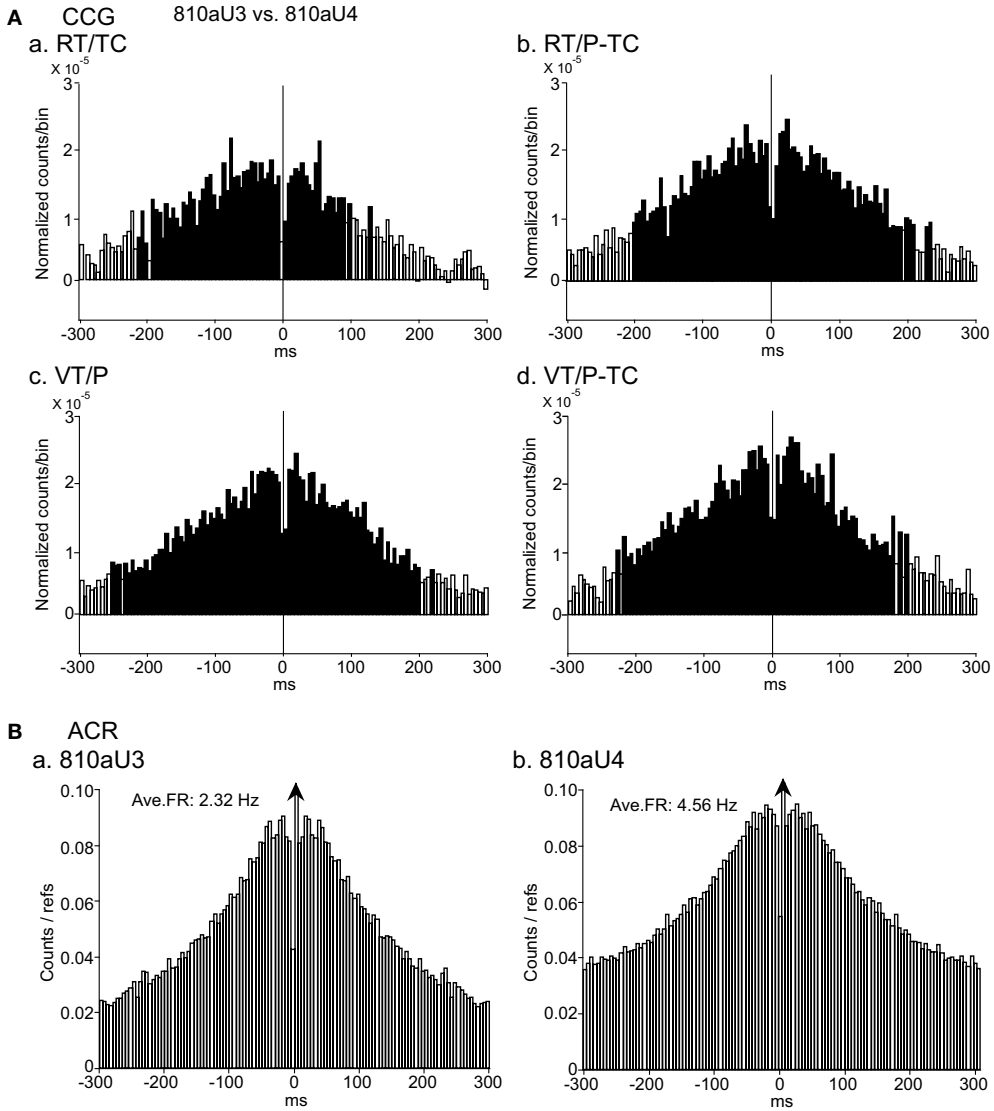

FIGURE 6 | An example of a neuron pair displaying task-independent positive peaks in the cross-correlograms (CCGs). (A) Difference CCGs (±300 ms binning range, 5 ms bin width) for a pair of CA1 pyramidal neurons (810aU3, reference cell; 810aU4, target cell). Note that symmetrical positive CCG peaks were observed in all four tasks **(a–d)**. Total spikes analyzed in each CCG in each task for neurons 810U3 and 810U4 were 2888 and 5584 spikes in the RT/TC, 2520 and 5002 spikes in the RT/P-TC, 2672 and 5190 spikes in the VT/P, and 2048 and 4552 spikes in the VT/P-TC tasks, respectively. **(B)** Autocorrelograms (ACRs; ±300 ms binning range, 5 ms bin width) of the same neurons in **(A)** [**(a)** 810aU3; **(b)** 810aU4]. Both neurons showed a similar discharge pattern in the ACRs. Other descriptions as for **Figure 4**.

were similar; inhibition around their discharges with long duration (about ±50 ms; **Figure 7B**). Four of five pairs with the negative CCG peaks, which were observed in all three types of neuron pairs, displayed task-independent correlation without a peak shift.

Figure 8A shows a typical example of task-independent center-negative and surround-positive CCG peaks between a pair of CA1 pyramidal neuron (501U1, reference cell) and interneuron (501U2, target cell). Both center-negative and surround-positive peaks were symmetrical at time zero and task-independent. The significant negative peaks were narrower than those in the pair of **Figure 7A** (about ±10 ms), and the significant positive peaks had broad bases ranging from −250 to +250 ms. The patterns of ACRs of these neurons (501U1 and 501U2) were similar (**Figure 8B**); inhibition around their discharges (i.e., time zero in the ACR histograms) lasted for a relatively short duration (about ±20 ms). Eleven of 14 pairs with the center-negative and surround-positive CCG peaks, which were comprised of all three types of neuron pairs, showed task-independent correlations, and 13 of these 14 pairs had no peak shift in the CCR histograms.

THREE HZ OSCILLATION IN CCGs

A few oscillatory activities were observed in the CCR histograms (3/98 pairs). An example of an oscillatory CCG peak between a pair of CA1 pyramidal neurons (811U1 and 811U2) in RT/TC task is

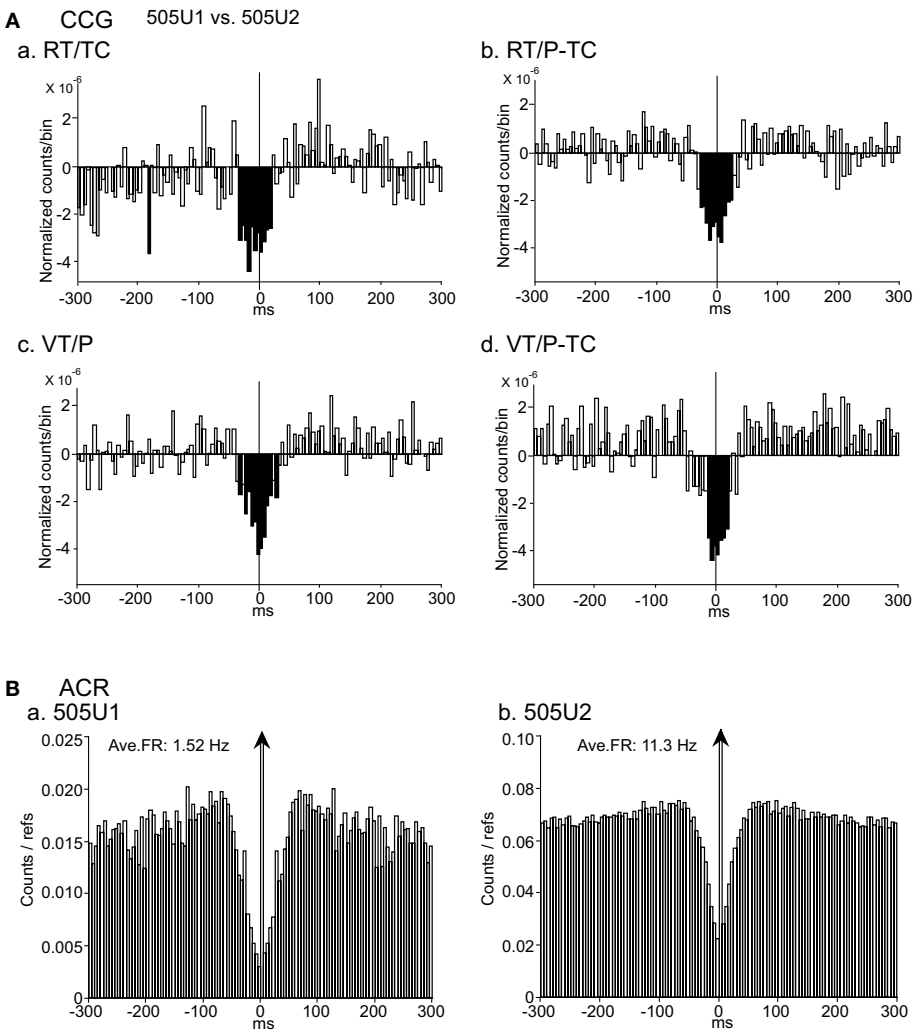

FIGURE 7 | An example of a neuron pair displaying task-independent negative peaks in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neurons (505U1, reference cell; 505U2, target cell) in the four tasks (waveforms are shown in **Figures 3Da,b**). Note that broad symmetrical negative peaks were observed in the four tasks **(a–d)**. Total spikes analyzed in each CCG in each task for neurons 505U1 and 505U2 were 2873 and 13900 spikes in the RT/TC, 1204 and 10187 spikes in the RT/P-TC, 1524 and 14661 spikes in the VT/P, and 1145 and 8365 spikes in the VT/P-TC tasks, respectively. **(B)** ACRs of the same neurons in **(A)** [**(a)** 505U1; **(b)** 505U2]. Other descriptions as for **Figure 4**.

shown in **Figure 9A**. It is noted that this pair displayed the positive CCG peak with a peak shift and this CCG peak was task-dependent. This task-selectivity was dependent on the firing rates of both neurons 811U1 and 811U2 (data not shown). ACR analysis indicated clear 3 Hz oscillation in 811U1 (**Figure 9Ba**), but not in 811U2 (**Figure 9Bb**). Thus, the oscillation observed in the CCR histogram of this pair was attributed to oscillatory activity of the neuron 811U1. All three pairs with the oscillatory CCG peaks displayed 3 Hz oscillation, and were comprised of pyramidal neuron vs. pyramidal neurons. Two of these three pairs had task-independent CCG peaks and the remaining one had the task-dependent CCG peak.

TRIGGER-LIKE NEURONAL DISCHARGES WITH TASK-DEPENDENCY

Three neuron pairs displayed interesting CCR histograms, in which one neuronal discharge (503U1) preceded activity of another neuron (503U3; **Figure 10A**, waveforms are shown in **Figure 3F**). Significant positive CCG peaks between the CA1 pyramidal neurons occurred 5 ms after time zero and lasted for more than 300 ms. It is noted that the positive CCG peak was task-selective and dependent on the average firing rate of both neurons 503U1 and 503U3 (data not shown). ACRs of neurons 503U1 (**Figure 10Ba**) and 503U3 (**Figure 10Bb**) showed bell-shaped peaks, but the peak was steeper in 503U1 neuron. All of these three pairs showed same characteristics, i.e., combination of pyramidal vs. pyramidal neurons, the firing rate-dependent task-selectivity, and peak shift.

RELATIONSHIP BETWEEN CCG PEAKS AND SPATIAL CORRELATES

To analyze the relationships between the CCG peaks and spatial correlates of the HF neurons, firing rate maps (Matsumura et al., 1999) were created. **Figure 11** illustrates the firing rate maps of a pair of 529U1 and 529U2 neurons, which showed

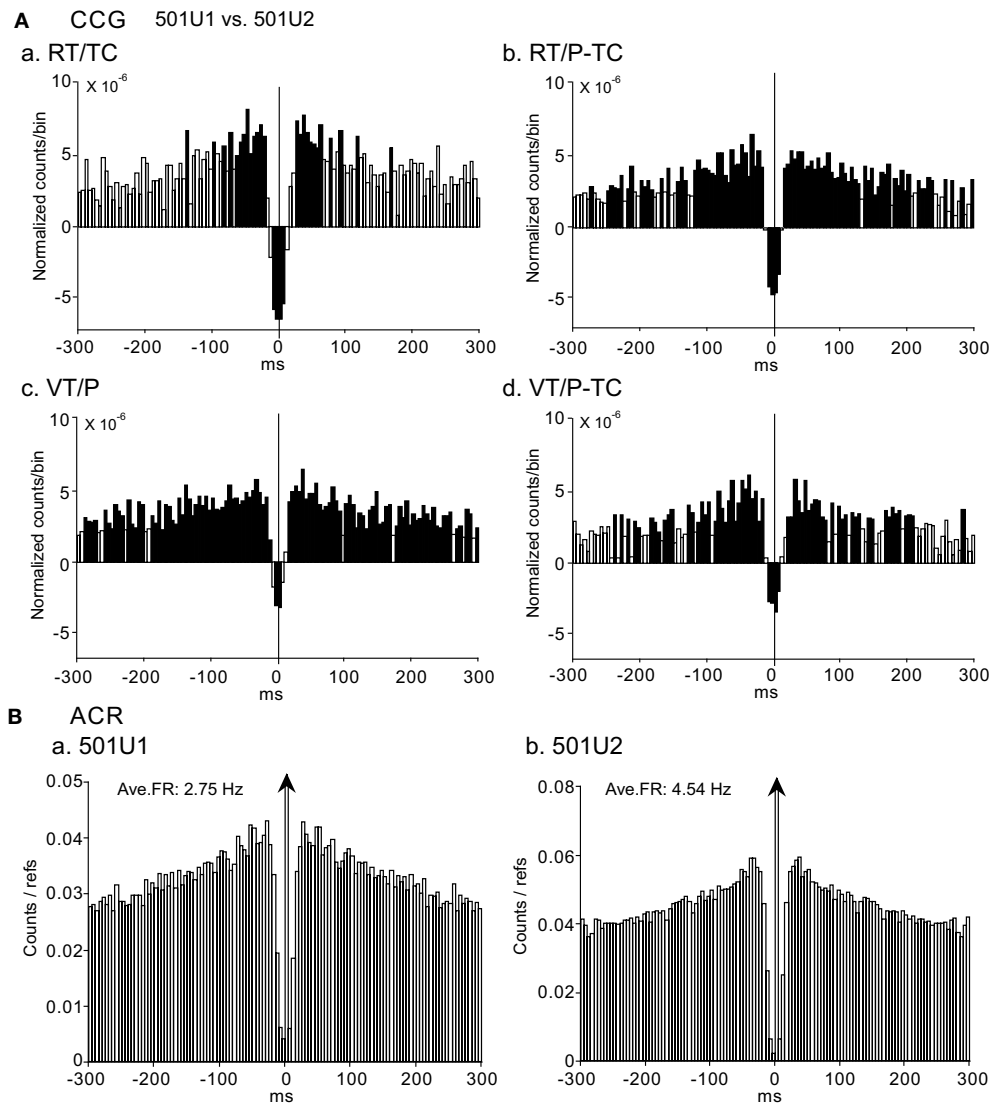

FIGURE 8 | An example of a neuron pair displaying task-independent center-negative and surround-positive peaks in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neuron (501U1, reference cell) vs. interneuron (501U2, target cell) in the four tasks **(a–d)**. Total spikes analyzed in each CCR in each task for neurons 501U1 and 501U2 were 1714 and 6623 spikes in the RT/TC, 3763 and 5698 spikes in the RT/P-TC, 3027 and 6273 spikes in the VT/P, and 1062 and 2659 spikes in the VT/P-TC tasks, respectively. **(B)** ACRs of the same neurons in **(A)** [**(a)** 501U1; **(b)** 501U2). Other descriptions as for **Figure 4**.

task-dependent CCRs in the VT/P-TC task (**Figure 4**). Neuron 529U1 displayed a place field only in the VT/P-TC task while neuron 529U2 displayed place fields in the RT/TC, RT/P-TC, and VT/P-TC tasks. It is noted that activity of the both neurons increased when the monkey visited around the center of the experimental field in the VT/P-TC task, so the place fields of these neurons overlapped only in the VT/P-TC task. When data sample was confined to place-differential neurons with the place fields, most neuron pairs with significant CCRs displayed overlap of the place fields in those tasks in which significant CCRs were observed; 66.7% (20/30) of the neuron pairs with task-dependent CCRs, and 73.2% (30/41) with task-independent CCRs displayed overlap of the place fields.

POPULATION ANALYSIS OF TASK-DEPENDENT CROSS-CORRELATION

Numbers of the neuron pairs with task-dependent (44 pairs) and independent CCRs (54 pairs) in each task were shown in **Figure 12A**. There was no significant difference in ratio of task-dependent and independent CCRs among four tasks (χ^2-test, $P > 0.05$). This indicates that there is no apparent difference of the number of neuronal populations relating task-dependency among the tasks. Next, to test strength of coactivation of the task-dependent neuronal populations, mean normalized counts between –50 and +50 ms were compared between task-dependent significant and non-significant CCRs by two-way analysis of variance (ANOVA; task × CCR significance; **Figure 12B**). The results indicated that there was a significant main effect of CCR significance [$F(1, 154) = 31.584$, $P < 0.0001$],

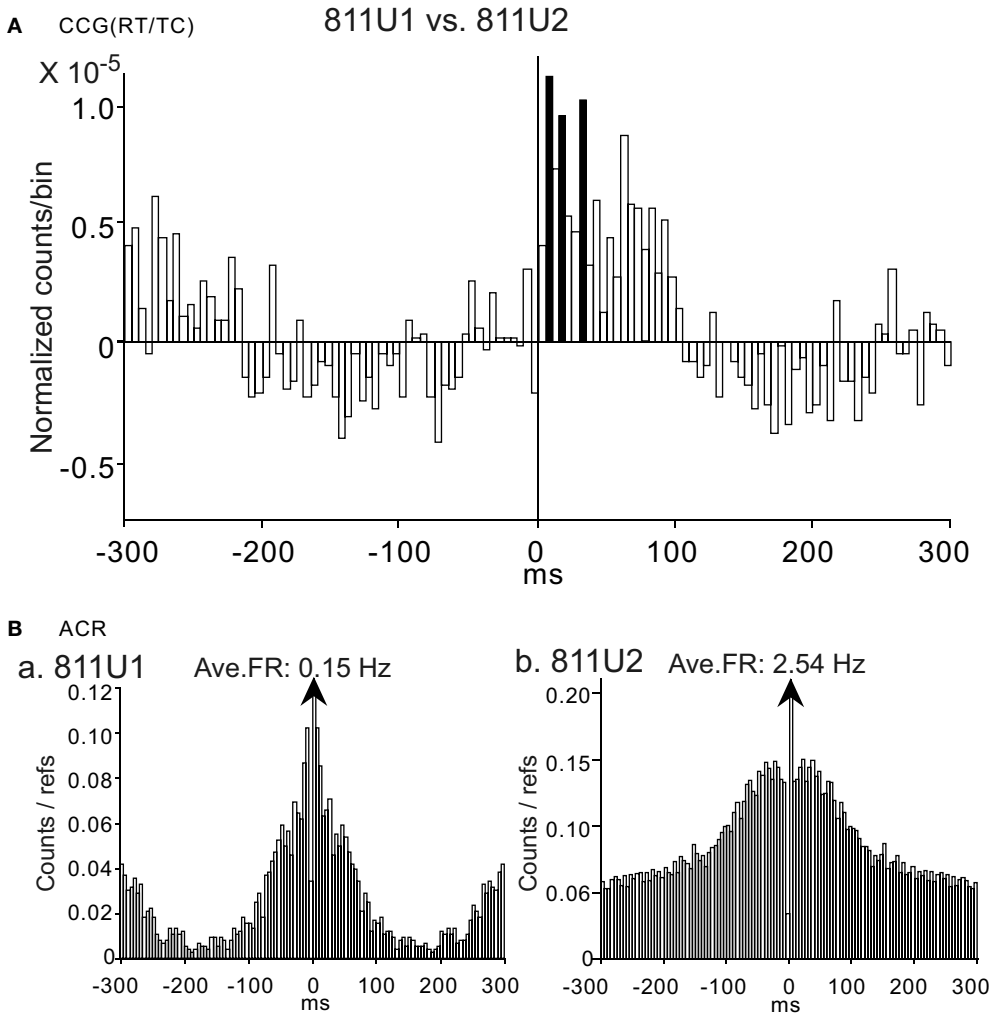

FIGURE 9 | An example of a neuron pair displaying a task-dependent peak with theta oscillation in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neurons (811U1, reference cell; 811U2, target cell) displaying an oscillatory CCG peak in the RT/TC task. Total spikes analyzed in each CCG in each task for neurons 811U1 and 811U2 were 804 and 5131 spikes in the RT/TC, 27 and 1440 spikes in the RT/P-TC, 14 and 231 spikes in the VT/P, and 14 and 374 spikes in the VT/P-TC tasks, respectively. **(B)** ACRs of the same neurons in **(A)** [**(a)** 811U1; **(b)** 811U2) indicating 3 Hz oscillation in 811U1 **(a)**, but not in 811U2 **(b)**. Other descriptions as for **Figure 4**.

while there was no interaction between task and CCR significance [$F(3, 154) = 0.746$, $P > 0.05$]. This indicates that specific populations of neuron pairs contribute to generation of task-dependent CCG peaks.

RELATIONSHIPS AMONG TASK-DEPENDENCY, PEAK LATENCY, AND PEAK POLARITY

Figure 13 summarizes the number of neuron pairs whose CCG peaks occurred at time difference. All peaks occurred within 50 ms time difference between discharges of neuron pairs, and they were observed most frequently at 0 (i.e., no peak shift) and within 10 ms latency.

Numbers of significant CCRs in each type of neuron pair are shown in terms of peak shift and peak polarity (left columns) in **Figure 14A**. There were some significant relationships among these parameters (Fisher's exact probability test, $P < 0.05$ in all); (1) ratio of overall positive CCG peaks (79/163) was larger than those of negative peaks (5/163) and center-negative and surround-positive peaks (14/163), (2) ratio of positive CCG peak was larger in pyramidal vs. pyramidal neuron pairs (67/163) than those in pyramidal neuron vs. interneuron (11/163) and interneuron vs. interneuron pairs (1/163), (3) ratio of CCG peaks with peak shift (69/163) was larger than that of CCG peak without peak shift (29/163), (4) ratios of positive CCG peaks with peak shift were much larger in pyramidal neuron vs. pyramidal neuron pairs (57/163) than pyramidal neuron vs. interneuron (8/163), and none in interneuron vs. interneuron (0/163) pairs. These results indicate that pyramidal vs. pyramidal neuron pairs with positive CCG peaks and peak shift predominate in the monkey HF and PH.

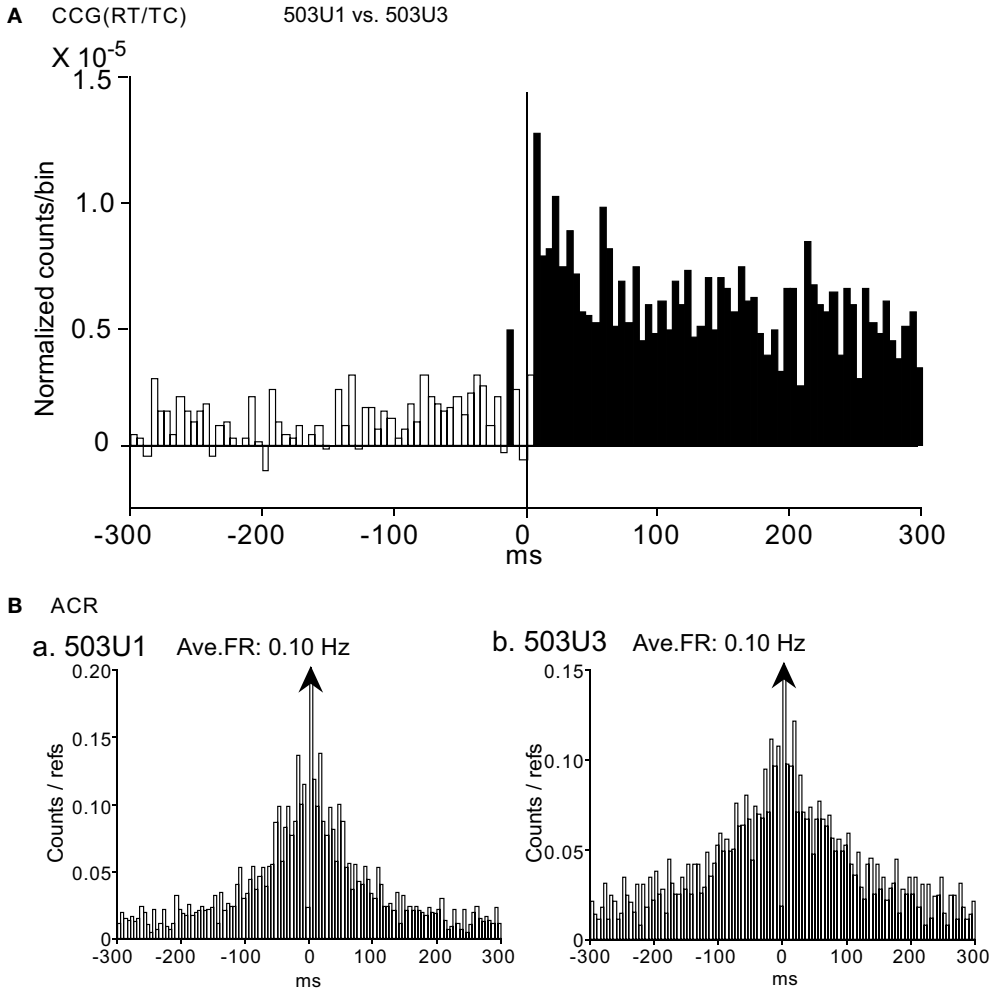

FIGURE 10 | An example of a neuron pair displaying a task-dependent peak with trigger-like activity in the CCGs. (A) Difference CCGs for a pair of CA1 pyramidal neurons (503U1, reference cell; 503U3, target cell) in the RT/TC task. Note that one neuronal discharges (503U1) precede activity of the other neuron (503U3). Total spikes analyzed in each CCG in each task for neurons 503U1 and 503U3 were 393 and 1837 spikes in the RT/TC, 33 and 819 spikes in the RT/P-TC, 26 and 383 spikes in the VT/P, and 78 and 1145 spikes in the VT/P-TC tasks, respectively. **(B)** ACRs of the same neurons in **(A)** [**(a)** 503U1; **(b)** 503U3]. Other descriptions as for **Figure 4**.

In contrast, ratio of negative CCG peaks (negative peaks and center-negative plus surround-positive peaks) were more common in the neuron pairs without peak shift (15/163) than those with peak shift (4/163; Fisher's exact probability test, $P < 0.05$; **Figure 14A**).

Task-dependency of significant CCG peaks in each type of neuron pairs with and without peak shift is summarized in **Figure 14B**. There were some significant relationships among these parameters (Fisher's exact probability test, $P < 0.05$); (1) the task-dependent CCG peaks were found only in pyramidal vs. pyramidal neurons (38/163) and pyramidal neuron vs. interneuron neuron (6/163) pairs, but none in interneuron vs. interneuron pairs (0/163), (2) ratios of task-dependent CCG peaks were more prominent in the neuron pairs with peak shift (35/163) than the neuron pairs without peak shift (8/163); (3) in pairs without peak shift, task-independent CCG peaks (21/163) were more common than that of task-dependent CCG peaks (8/163), (4) task-dependent CCG peaks were more common in pyramidal vs. pyramidal neuron pairs with peak shift (30/163) than pyramidal neuron vs. interneuron (5/163) and interneuron vs. interneuron (0/163) pairs with peak shift, (5) there were no significant differences in ratios of neuron pairs with peak shift between pyramidal vs. pyramidal neuron pairs with task-dependent (30/163) and task-independent (31/163), (6) there were no significant differences in ratios of task-independent CCG peaks between neuron pairs without (21/163) and with (34/163) peak shift. These data indicate that pyramidal vs. pyramidal neuron pairs with positive CCG peaks and peak shift contribute to both task-dependent and independent information processing, and that neuron pairs both with and without peak shift contribute to task-independent information processing.

Overall, these results suggest some important characteristics of functional connectivity in the HF that pyramidal neuron vs. pyramidal neuron pairs with positive peak and peak shift

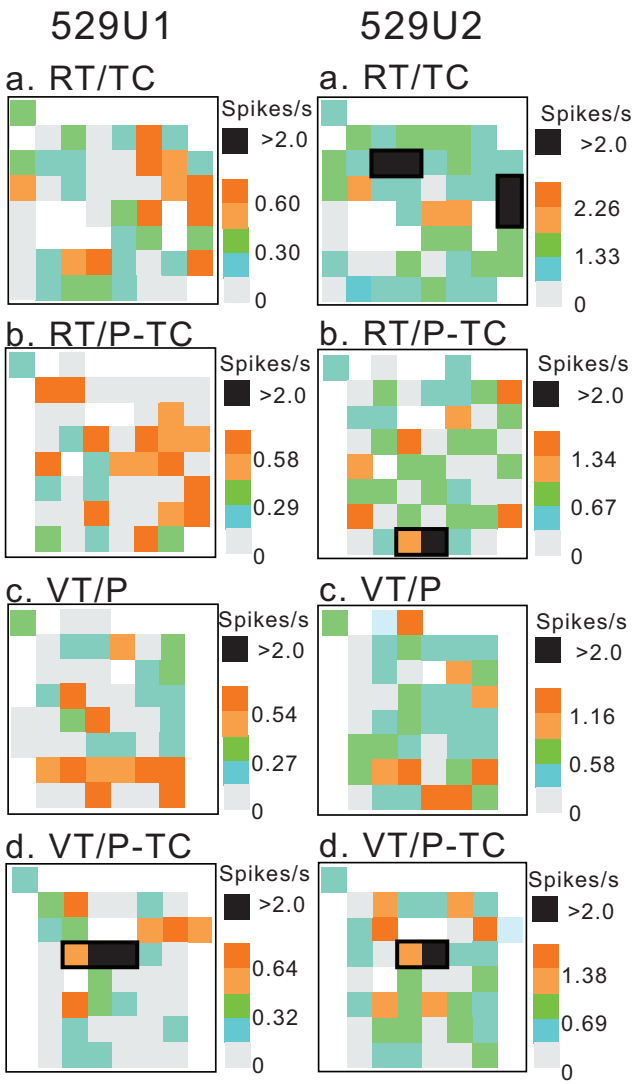

FIGURE 11 | Firing rate maps of a pair of neurons 529U1 and 529U2 which displayed a task-dependent CCR in the VT/P-TC task shown in Figure 4. Place fields are surrounded by thick lines. Within the place fields, pixels with the firing rates greater than 2.0 spikes/s are indicated by filled squares. Note that place fields of the both neurons overlapped in the VT/P-TC task. Calibration is shown in right; a mean firing rate for each pixel was expressed as a relative firing rate (R) in which the mean firing rate in each pixel was divided by the grand mean firing rate (M) in each task, and shown in five steps ($R \geq 2M$; $2.0M > R \geq 1.5M$; $1.5M > R \geq 1.0M$; $1.0M > R \geq 0.5M$; $R < 0.5M$). Three values in the calibration indicate those of $2M$, M, and 0, respectively. Regions not visited by the monkey during the session(s) are shown by blank pixels.

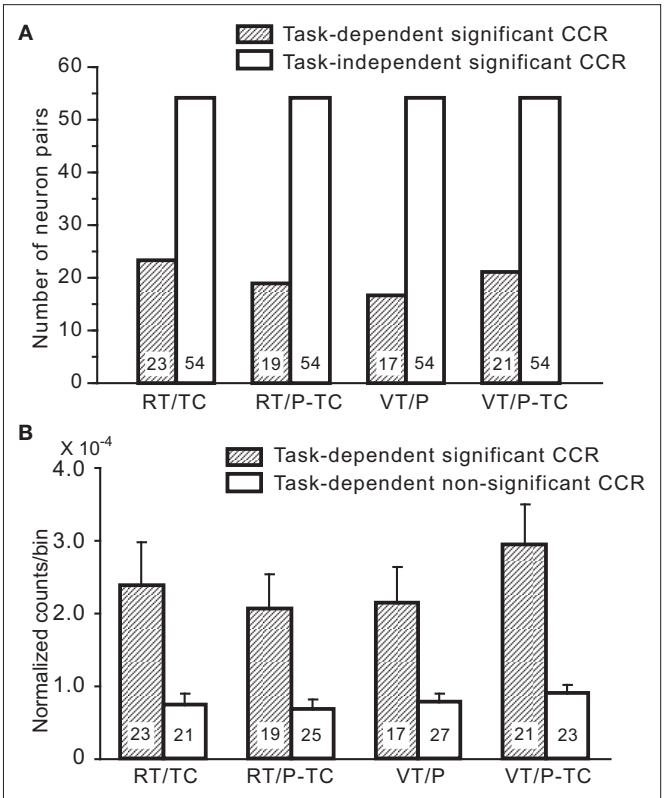

FIGURE 12 | Characteristics of the neuron pairs with the task-dependent CCG peaks. (A) Frequency histogram of task-dependent and task-independent significant CCG peaks in each task. By definition, the same numbers of task-independent significant CCRs were found in all tasks. **(B)** Comparison of mean normalized counts between –50 and +50 ms in the CCRs between task-dependent significant and non-significant CCG peaks. Numbers of the neuron pairs are indicated in each column.

contribute to task-dependent information processing. However, no consistent patterns were observed in task-independent CCG peaks that both pyramidal neuron vs. pyramidal neuron and interneuron vs. interneuron pairs contribute to task-independent information processing, and that both neuron pairs with and without peak shift contribute to task-independent information processing.

REGIONAL DIFFERENCES AMONG SUBREGIONS IN THE HF AND PH

Numbers of the significant and non-significant CCRs based on neuron pair types in each subregion of the HF and PH are shown in **Table 1**. Characteristics of the neuron pairs with significant CCRs in each subregion of the HF and PH regarding task-selectivity, peak shift, and peak polarity are shown in **Table 2**. Although most neuron pairs were recorded from the CA1 subregion and dentate gyrus, the trends of CCR characteristics in each subregion were similar to those noted in the above section; (1) pyramidal vs. pyramidal neuron pairs predominated in the all subregions, (2) neuron pairs with peak shift predominated in the all subregions, and (3) neuron pairs with positive peak polarity predominated in the all subregions.

DISCUSSION

GENERAL CHARACTERISTICS

In the present study, multiple single units were recorded from a same single electrode. Relatively high incidence of significant CCRs in the present study might be attributed to recording of neighboring neurons, consistent with previous studies (Sakurai, 1996; Takahashi and Sakurai, 2009). Strength of correlation in CCRs depended on the distance between two neurons in the auditory

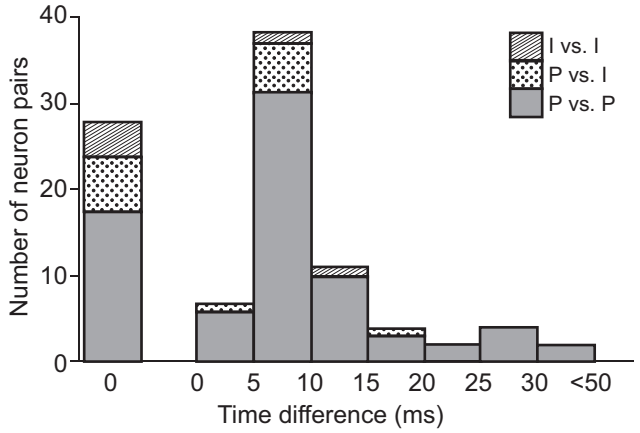

FIGURE 13 | Frequency histogram of the time differences of the CCG peaks in terms of the types of the neuron pairs. P vs. P, pyramidal neuron vs. pyramidal neuron; P vs. I, pyramidal neuron vs. interneuron; I vs. I, interneuron vs. interneuron.

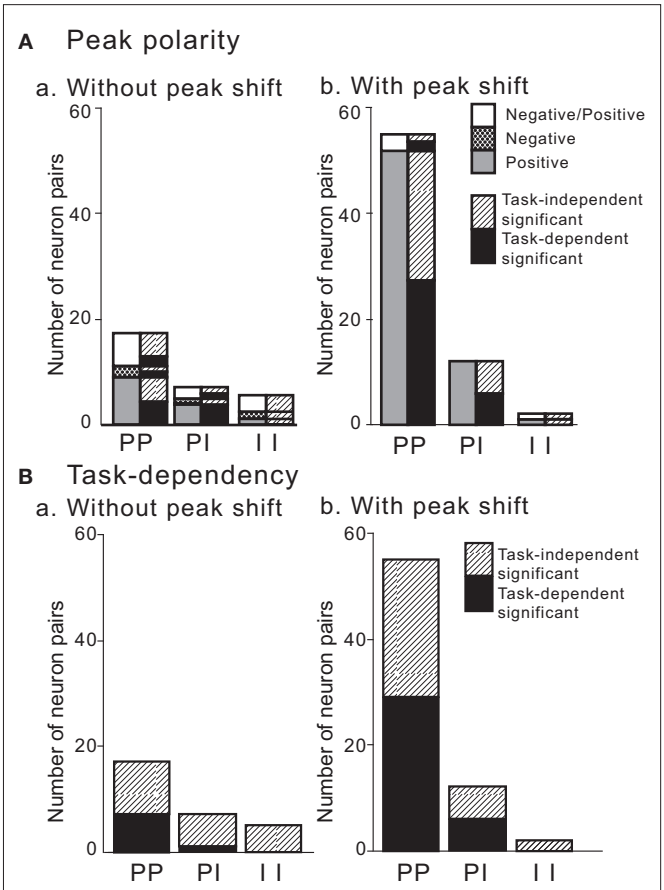

FIGURE 14 | Polarity (A) and task-dependency (B) of the CCG peaks without or with a peak shift in terms of the types of the neuron pairs. To facilitate comparison of the data between **(A)** (peak polarity) and **(B)** (task-dependency), corresponding task-dependency is shown in the right column of each type of neuron pair in **(A)**. Note that most pairs with a peak shift had positive peaks. Positive, positive peaks; Negative, negative peaks; Negative/Positive, center-negative and surround-positive peaks. PP, pyramidal neuron vs. pyramidal neuron; PI, pyramidal neuron vs. interneuron; II, interneuron vs. interneuron. Task-independent significant, task-independent significant CCG peaks; task-dependent significant, task-dependent significant CCG peaks.

cortex (Maldonado and Gerstein, 1996). The neighboring neurons in the auditory cortex had better synchronization (Eggermont and Smith, 1996).

Most neuron pairs with a peak shift displayed long discharge time differences (14 ms on average). This suggests that direct (i.e., monosynaptic) interaction was not obvious between nearby neurons. These results are consistent with the previous study in which there was no evidence indicating direct connection between CA1 pyramidal neurons nearby in the intracellular recordings *in vitro* (Nakajima et al., 1991).

TASK-DEPENDENT SYNCHRONOUS ACTIVITY

The four tasks used in the present study had different characteristics. In the RT/TC task, the pointer in the monitor was not presented and the monkeys always faced in a fixed direction. This indicated that the monkey could recognize same landmarks in each trial, and suggests that a cognitive map, in which spatial relationships of the landmarks are represented, is required for the performance of RT/TC task. In the RT/P-TC task, however, they did not necessarily judge their position based on any cognitive map since the position was indicated by the pointer on the monitor. The VT (VT/P and VT/P-TC) tasks required the monkey to move a pointer to a target area on the monitor, and required no cognitive map system.

The task-dependent neural correlates in the HF were reported previously (Matsumura et al., 1999). In the present study, the synchronous activity in the HF and PH was also altered in a task-dependent manner with or without dependence on the firing rate. This task-dependent synchronous activity was most frequently found in the neuron pairs of pyramidal vs. pyramidal neurons, while the synchrony between interneurons was always task-independent. Furthermore, these neuron pairs with task-dependent CCGs displayed peak shift with positive peaks. These results suggest that information for specific tasks is encoded by the asymmetrical neural circuits connecting pyramidal neurons, and that interneurons are involved in task-independent common information across the tasks. Asymmetrical connection between pyramidal neurons has been suggested to be a neurophysiological basis in sequence learning and memory in the rat HF (Skaggs et al., 1996; Lisman, 1999; Nadásdy et al., 1999; Sato and Yamaguchi, 2003; Wu and Yamaguchi, 2004). The present study further indicates that the asymmetrical neural circuits connecting pyramidal neurons are important in encoding of task-specific information as noted below, consistent with a computational study (Levy, 1996; Wagatsuma and Yamaguchi, 2007).

The task-dependent synchronous activity was found in both real and VT tasks, and there was no clear difference in the CCG peaks between the tasks. This result indicates that the task-dependent functional connectivity of the HF neurons may be produced in any kind of tasks. This also suggests that the monkey HF does not strictly contribute to spatial behaviors, but is involved in encoding of other factors such as task-specific information. It has been reported that about 40% of the monkey HF neurons

Table 1 | Number of neuron pairs showing significant and non-significant cross-correlograms (CCGs) in each subregion of the HF and PH.

		Type of neuron pairs				
Subregion	**Signif-icant**	**P vs. P**	**P vs. I**	**I vs. I**	**Subtotal**	**Total**
CA1	–	23	3	0	26	84
	+	48	6	4	58	
CA3	–	0	0	0	0	1
	+	1	0	0	1	
DG	–	5	15	5	25	47
	+	14	5	3	22	
SUB	–	8	0	2	10	20
	+	8	2	0	10	
PH	–	4	0	0	4	11
	+	7	0	0	7	
Subtotal	–	46	14	5	65	
	+	72	18	8	98	
Total		118	32	13	163	

P vs. P, Pyramidal vs. pyramidal neurons.
P vs. I, Pyramidal neuron vs. interneuron.
I vs. I, Interneuron vs. interneuron.
DG, dentate gyrus; SUB, subiculum; PH, parahippocampal gyrus.
+, significant CCR; –, non-significant CCR.

Table 2 | Characteristics of the neuron pairs with significant CCRs in each subregion of the HF and PH regarding task-selectivity, peak shift, and peak polarity.

	Task-selectivity		**Peak shift**		**Peak polarity**		
	+	–	+	–	**P**	**N**	**NP**
CA1	24	34	40	18	49	3	6
CA3	0	1	1	0	1	0	0
DG	10	12	12	10	14	1	7
SUB	4	6	10	0	9	0	1
PH	5	2	6	1	6	1	0

Each number indicates number of neuron pairs in each category. Task-selectivity (+), task-dependent CCR; task-selectivity (–), task-independent CCR; peak shift (+), CCRs with peak shift; peak shift (–), CCRs without peak shift; P, positive; N, negative; NP, center-negative and surround-positive peaks. Other descriptions as for ***Table 1****.*

showed place-differential activity, which were sensitive to tasks (i.e., task-dependent; Matsumura et al., 1999). The authors suggested that an assembly of reference frames may be a neural basis of episodic memory for various events. Consistently, rodent HF place cells were sensitive to specific reference frames (Samsonovich and McNaughton, 1997; Skaggs and McNaughton, 1998; Tabuchi et al., 2003). HF place cells in old rats displayed inconsistency to same reference frames, relating to cognitive and memory deficits (Barnes et al., 1997). The present finding of the task-dependent CCG peaks suggests that different neural circuits may be active for task-dependent memory. Taken together, it is strongly suggested that the HF neural circuits may be important for representation of specific information for the different tasks, and that neuronal populations selective to task situation or memory may originate in the HF.

TASK-INDEPENDENT SYNCHRONOUS ACTIVITY

All the three types of pairs (PP, PI, II) displayed task-independent CCG peaks. Data in **Figures 6 and 7** suggest that the neuron pairs received common inputs as noted below. The positive and task-independent CCG peaks were observed in the neuron pairs of pyramidal vs. pyramidal neurons (**Figure 6**) and pyramidal neuron vs. interneuron. In this case, common excitatory inputs for long duration (>300 ms) could promote discharges of both types of neuron pairs. Negative CCG peaks shown in **Figure 7** also suggest involvement of common inputs. This pattern was observed mainly in the neuron pairs of pyramidal neuron vs. interneuron. In addition, all pairs had no clear peak shift, and CCG in all pairs except one were task-independent. The possible circuits might involve inhibitory inputs from upstream inhibitory neurons that could suppress activity of both a pyramidal neuron and an interneuron. Center-negative surround-positive CCG peaks (**Figure 8**) were observed in all three types of neuron pairs. The majority of the pairs had no clear peak shift and their peaks were task-independent. More complex neural circuits including the inhibitory inputs could be involved in this case.

The GABAergic innervation from the medial septum and the nucleus of the diagonal band, which is a major source of theta rhythm in the HF, inhibits or disinhibits the pyramidal neurons through interneurons within the HF (Stewart and Fox, 1990). The cholinergic system from the same brain regions excites directly the pyramidal neurons or interneurons (Stewart and Fox, 1990). These inhibition and excitation mechanisms in the HF might contribute to form CCG peaks without a peak shift implicating common inputs. The common inputs might convey task-independent information such as arousal and intention, which were supposed to be similar across the tasks, since behavioral requirement (i.e., joystick manipulation), reward (juice), and behavioral performance were similar across the tasks. It has been reported that there are several types of interneurons in the HF based on the morphology and pharmacology (Freund and Buzsáki, 1996; Halasy et al., 1996). Unidirectional and reciprocal inhibitory interneurons connect with pyramidal neurons (Stewart and Fox, 1990). The local interneurons within the HF could modulate discharge patterns to generate complex CCG peaks as well as oscillations in the ACRs. Further studies are necessary to elucidate the neural circuits in the HF.

THETA OSCILLATION AND TRIGGER-LIKE ACTIVITY

Oscillatory activity was involved in the synchronization between the HF neurons in primates (**Figure 9** and weakly in **Figures 4 and 5**), consistent with the data in rodents (Mizumori et al., 1990; Skaggs et al., 1996; Tabuchi et al., 2000). However, there were some differences between primate and rodent data. First, frequency of the theta oscillation was low at 3 Hz in the present study. This frequency is consistent with the electroencephalogram (EEG) data at 3–12 Hz in monkeys (Stewart and Fox, 1990) and humans (Kahana et al., 1999), but lower than 9 Hz in rodents (Tabuchi et al., 1999). Second, theta oscillation was not common in monkeys (3/98 pairs in the present study). Third, theta rhythm was observed only between

the pyramidal neurons. These data are inconsistent with those in rats in which the theta oscillation was frequently found in both pyramidal neurons and interneurons (Mizumori et al., 1990; Skaggs et al., 1996; Tabuchi et al., 1999). These suggest different roles of theta oscillation between primates and rodents.

In three pairs of pyramidal vs. pyramidal neurons, discharges of one neuron were followed by those of the other neurons with high frequency burst (**Figure 10**). This indicates that the pyramidal neurons of these three pairs can be separated into two types; trigger type neurons with low frequent discharges and bursting neurons. The CCRs for these pairs were task-dependent. The bursting neurons also displayed place-differential responses. These results suggest that these neuron types might be involved in encoding of task-dependency by induction of long-term potentiation in which high frequency activation plays an important role.

CONCLUSION

In the present study cross-correlation was examined between two neurons simultaneously recorded during performance of the four spatial tasks. The results indicate that functional connectivity of some HF neuron pairs changed in a task-dependent manner. These task-dependent CCRs were frequently observed in the pairs of pyramidal vs. pyramidal neurons. The time difference of their synchronous discharges suggested indirect asymmetrical connections between these neurons. These results support the idea that task-dependent neuronal networks in the HF encode context or reference frames, which might be a neural basis for episodic memory. In contrast, the task-independent CCRs were observed in all three types of neuron pairs. This suggests involvement of non-selective common inputs across the tasks such as arousal or intentional inputs to both pyramidal neurons and interneurons in the HF. These results suggest that the HF task-dependent neural circuits with asymmetrical connections are critical to disambiguously guide animals to places associated with reward in different situations.

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Sex-related memory recall and talkativeness for emotional stimuli

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Recent studies have evidenced an increasing interest in sex-related brain mechanisms and cerebral lateralization subserving emotional memory, language processing, and conversational behavior. We used event-related-potentials (ERP) to examine the influence of sex and hemisphere on brain responses to emotional stimuli. Given that the P300 component of ERP is considered a cognitive neuroelectric phenomenon, we compared left and right hemisphere P300 responses to emotional stimuli in men and women. As indexed by both amplitude and latency measures, emotional stimuli elicited more robust P300 effects in the left hemisphere in women than in men, while a stronger P300 component was elicited in the right hemisphere in men compared to women. Our findings show that the variables of sex and hemisphere interacted significantly to influence the strength of the P300 component to the emotional stimuli. Emotional stimuli were also best recalled when given a long-term, incidental memory test, a fact potentially related to the differential P300 waves at encoding. Moreover, taking into account the sex-related differences in language processing and conversational behavior, in the present study we evaluated possible talkativeness differences between the two genders in the recollection of emotional stimuli. Our data showed that women used a higher number of words, compared to men, to describe both arousal and neutral stories. Moreover, the present results support the view that sex differences in lateralization may not be a general feature of language processing but may be related to the specific condition, such as the emotional content of stimuli.

Keywords: event-related-potential (ERP), P300 wave, language, gender, emotional stimuli, memory, talkativeness

INTRODUCTION

Emotional events are better memorized than neutral events and in recent years many animal and human studies have yielded evidence for it (Roozendaal, 2000; Arntz et al., 2005; Gasbarri et al., 2005, 2006, 2007, 2008a).

Recent studies have also revealed seemingly large, but previously unsuspected, sex-related influences on this mechanism (Frank and Tomaz, 2000; Cahill, 2006; Gasbarri et al., 2006, 2007; Hofer et al., 2007). For example, the role of the amygdala in emotional memory reveals a different sex-related hemispheric specialization: activity of the right, but not of the left, hemisphere amygdala relates significantly to memory of emotional material in men; conversely, activity of the left, but not the right, hemisphere amygdala relates significantly to memory of emotional material in women (Cahill, 2006). The evidence that each amygdala is likely to modulate information processing in other brain areas (McGaugh, 2000, 2004; Kilpatrick and Cahill, 2003), combined with the fact that amygdalo-cortical projections are almost exclusively ipsilateral, led us to consider the possibility that a sex-related hemispheric lateralization of processing for emotional material may also exist to some degree in cortical regions.

Moreover, despite an absence of sex differences in behavioral performance during the discrimination of emotional (sad and happy) vocal intonation, men evidenced significantly higher activation in the right middle frontal gyrus, while women showed higher activation in the left middle temporal gyrus (Wildgruber et al., 2002). Also, an effect of sex on brain activation during the recognition of fearful faces, despite no sex differences in task performance was reported (Kempton et al., 2009).

Sex differences in cognition are consistently reported: for example, men excel in mental rotation and spatial perception while women perform better in verbal memory tasks, verbal fluency tasks, speed of articulation, episodic memory tasks, and in the utilization of prosodic information (Schirmer et al., 2002; Schirmer and Kotz, 2003). Furthermore, language and reading disorders are reported to occur approximately twice as often in boys than in girls (Flannery et al., 2000), reflecting the sex-related difference in cognitive skills. Sex-related differences in language processing are well known from everyday life, as well as from scientific literature (Hill et al., 2006; Catani et al., 2007). Why females generally perform better on language tasks than males is still unknown (Burman et al., 2008). A common hypothesis attributes this differences to a bilateral contribution of language-related cerebral areas in females and a left-hemispheric dominated activation in males (Josse and Tzourio-Mazoyer, 2004; Sommer et al., 2004; Kitazawa and Kansaku, 2005; Ortigue et al., 2005; Hill et al., 2006; Ikezawa et al., 2008). Women might thus use both hemispheres for language functions, while men predominantly

use the left hemisphere. However, imaging studies reviewed by Sommer et al. (2007) failed to find such a generalized lateralization effect, and reported a left-lateralized activation in both sexes instead. Therefore, sex differences in lateralization may not be a general feature of language processing, but could be related to specific conditions, such as the chosen research method and the heterogeneity of the language tasks used (Hill et al., 2006). This topic is still debated and in the center of attention for many reasons (Clements et al., 2006; Plante et al., 2006). First, there are considerable differences between men and women in the development of language abilities. In fact, when speaking first begins, girls usually articulate better than boys and create longer sentences; perhaps, as a consequence, compared to boys, girls tend to have larger working vocabularies, better use of grammar, and superior reading abilities. Part of this verbal advantage for females survives into adult age, mainly in the domain of use of grammar and verbal fluency (Sommer et al., 2008). Moreover, studies on language disabilities both of severe and mild type reported that boys are affected more frequently than girls (Liederman et al., 2005). Some psychiatric disorders (e.g., autism, attention deficit hyperactivity disorder, schizophrenia) have higher prevalence in males than females (Afifi, 2007), and may be associated with deviations in standard cerebral dominance (Sommer et al., 2007). Additionally, after left cerebral stroke, women appear to recover from aphasia better than men (Pedersen et al., 1995). If a more bilateral language lateralization in women, compared to men, really exists, this could give an explanation for all these observed sex-related differences.

The aim of the present paper is to extend our previous findings, indicating that both sex and cerebral hemisphere constitute important interacting influences on neural correlates of emotion, and emotional memory (Gasbarri et al., 2006, 2007), and to analyze sex-related differences in talkativeness, while describing emotional stimuli.

MATERIALS AND METHODS

SUBJECTS

Sixty-four subjects (32 women and 32 men), mean age 25 (± 4.15), all University of L'Aquila students, viewed pictures from the International Affective Picture System (IAPS).

All the subjects were initially submitted to a screening interview to check for any health problems. All subjects were right-handed, had normal vision and were not suffering from any neurological disorder. Exclusionary criteria included any major medical or psychiatric illness, substance abuse, or history of head injury. All experiments were conducted in accordance with the declaration of Helsinki, and all the procedures were carried out with the adequate understanding of the subjects, who read and signed an informed consent before participating in this research study.

STIMULUS MATERIALS

The stimulus materials, utilized in this study, consist in emotional stimuli from the IAPS, a set of calibrated picture stimuli widely used in investigating emotion (Lang et al., 1999), previously employed in emotional memory studies (Cahill and McGaugh, 1995; Adolphs et al., 1997; Cahill, 2005; Gasbarri et al., 2005, 2006, 2007, 2008a; Satler et al., 2007).

The set of stimuli consisted in 60 pictures[1] selected from the IAPS (Lang et al., 1999), according to the valence dimension 20 pleasant, 20 unpleasant, and 20 neutral slides. Pleasant slides were puppies, happy babies, etc., unpleasant slides showed mutilations, wounded people, etc., and neutral slides were common things, such as household objects. More details on this kind of stimulus material can be found in other studies (Cahill and McGaugh, 1995; Adolphs et al., 1997; Frank and Tomaz, 2000; Tomaz et al., 2003; Cahill, 2005; Gasbarri et al., 2005, 2006, 2007, 2008a; Satler et al., 2007).

PROCEDURE

Participants viewed the IAPS pictures, while seated in a comfortable chair in a sound-attenuated, dimly lit room. After electrode attachment and laboratory adaptation, they were told that a series of emotional slides would be presented and that they should observe each picture the entire time it appeared on the screen, trying not to move their eyes. The entire projection of the slides lasted 10 min; each slide image was presented for 2 s, with the order of the slides randomized for each subject. The trigger was recorded at the beginning and at the end of the stimulus presentation. An 8 s interval occurred between each trial, during which the screen was completely black except for a small cross at the center of the screen which subjects were instructed to stare at. No cross was shown while the pictures were presented.

At the end of the recording procedures, the participants were asked to judge their personal emotional reactions to the pictures on a scale measuring affective valence. The values included in the scale ranged between 1 (indicating a very unpleasant image) and 10 (indicating a very pleasant image); a score of 5 indicated "not emotional." The subjects were instructed to come back to the laboratory after 10 days, but no mention of a memory test was made. When they came back, subjects performed a free recall task, in which they were asked to recall as many slides as possible, writing down a word or a short sentence describing each one. More details about this kind of stimulus material can be found in previous research (Cahill and McGaugh, 1995; Adolphs et al., 1997; Gasbarri et al., 2005, 2006, 2007, 2008a; Satler et al., 2007).

The free recall was recorded on a portable tape recorder; the number of words utilized in the free recall of positive, negative, and neutral stimuli was counted in female and male subjects, in order to evaluate possible talkativeness differences between the two sexes in the recollection of emotional stimuli.

ERP recording

EEG signals were recorded at 19 scalp sites (Fp1/Fp2, F3/F4, C3/C4, P3/P4, O1/O2, F7/F8, T3/T4, T5/T6, Cz, Fz, Pz), according to the International 10/20 System, (Lang et al., 1999) employing actiCap (Brain Products GmbH) with active electrodes. Impedance measurement displayed directly at the electrodes and active shielding is implemented; we used the actiCap Control Software (Brain Products GmbH).

[1]The IAPS slide numbers were as follows: neutral, 7170, 2200, 2210, 7000, 5510, 6150, 7040, 7050, 7080, 7090, 7100, 7130, 7150, 2190, 7500, 7550, 7560, 7700, 7710; pleasant, 1610, 1710, 1750, 1920, 1602, 2050, 2070, 2080, 2501, 2160, 2260, 2340, 1440, 4660, 5200, 5600, 5760, 5830, 7330, 7580; unpleasant, 9410, 2800, 3000, 3010, 3100, 2121, 3120, 3130, 2120, 9250, 3350, 3140, 3150, 3170, 3180, 3220, 6010, 6200, 6230, 9000.

Our EEG equipment (BRAINAMP – Brain Products GmbH) includes the Vision Recorder software, which measures specifically with very high precision GND (ground) and REF (reference) electrodes impedance. Impedance of each electrode was checked to insure it was ≤5 kΩ. Horizontal and vertical eye movements were monitored with a bipolar recording from electrodes at left and right outer canthi. The EEG from each electrode site was digitalized at 250 Hz. To further reduce high-frequency noise, the averaged visual evoked potentials were filtered at 0.01 Hz (24 dB/octave) and 30 Hz (24 dB/octave) with 50 Hz notch filter.

Visual evoked potentials were stored for offline averaging using Brain Vision Analyzer (Version 1.05.0001 – Brain Products GmbH). Epochs of averaging were 100 ms prior the stimulus onset and 1000 ms after stimulus onset.

Computerized artifact rejection was performed prior to signal averaging in order to discard epochs in which deviation in eye position, blinks, or amplifier blocking occurred. We used the difference criterion with the maximal allowed absolute difference of two values in the segment of 250.00 μV.

On average, about 18% of the trials were rejected for violating these artifact criteria. We considered "not valid" trials of the subjects who interrupted the EEG recordings for personal reasons and trials in which deviation in eye position, blinks, or amplifier blocking occurred during the averaging.

WORD COUNT

In order to evaluate possible talkativeness differences between the two sexes in the recollection of emotional stimuli, we counted the number of words that men and women used when they were submitted to a declarative memory test, using IAPS slides as emotional stimuli. To this aim, research assistants listened to audio-recordings of the task and tallied the words with an electronic counter. According to other studies (Wardle et al., 2011), non-word utterances such as "umm," "uhh," "err," and "ehh" were included in the count. The decision to include non-word utterances was made on the basis that these "filled pauses" indicate intent or urge to speak, and thus should be treated differently than silence. Indeed, in previous research, filled pauses have been particularly sensitive to drug effects (Epstein et al., 2010; Marrone et al., 2010), suggesting that they do differ qualitatively from unfilled pauses. A 30-s segment of the task of each subject was recounted by a "standard" rater (the first author). If his count of the sample differed by more than two words from the count given by a research assistant, the entire recording was recounted. This procedure resulted in less than 1% of individual tasks needing to be recounted, and none of these final counts were discrepant from the original counts by more than 10 words.

STATISTICAL ANALYSIS

Data obtained were analyzed using analyses of variance (ANOVA). The analysis of event-related-potential (ERP) parameters, P300 amplitude, and latency was conducted separately. All trials that violated the artifact criteria were rejected. Differences between groups were tested using Tuckey's test.

Statistical significance was set at $p < 0.05$. All data are presented as means ± SEM.

RESULTS

EMOTIONAL REACTIONS

The personal emotional responses to the IAPS slides reliably discriminated neutral from emotional pictures, and confirmed differences in affective content between the slide categories. Unpleasant pictures were judged significantly less pleasant than the neutral pictures which, in turn, were rated less positively than pleasant pictures.

The three stimulus contents were distinct and representative of affect type: mean valence (nine-point scales, pleasant high) for pleasant pictures was 7.11 (±1.34) in men and 7.65 (±0.89) in women. Mean valence for unpleasant pictures was 2.74 (±1.26) in men and 2.39 (±1.35) in women. Mean valence for neutral pictures was 5.20 (±0.86) in men and 5.04 (±1.17) in women.

EVOKED POTENTIALS

The P300 window was set at 300–450 ms after stimulus onset. P300 was quantified within each average waveform as the largest positive deflection in that epoch. Both P300 amplitude and latency data were computed. Grand-average ERPs in response to unpleasant, pleasant and neutral stimuli are presented in **Figure 1**.

P300 amplitude

The P300 amplitude was determined for F3/F4, and P3/P4 electrode sites in both men and women (**Figure 2**). All data were analyzed using ANOVA followed by Tuckey's test.

Frontal sites. At site F3, women showed a P300 amplitude in response to unpleasant slides that was significantly higher compared to both pleasant [$F(1,62) = 35.25$; $^{*}p < 0.0001$] and neutral slides [$F(1,62) = 156.02$; $^{**}p < 0.001$]. Moreover, in women the P300 amplitude in response to unpleasant slides was significantly higher compared to men [$F(1,62) = 40.89$; $\bullet p < 0.0001$].

In men, P300 amplitude in response to unpleasant slides was significant higher at site F4 compared to F3 [$F(1,62) = 9.40$; $^{*}p < 0.003$], while in women it was significantly higher at site F3 compared to F4 [$F(1,62) = 22.26$; $\blacklozenge p < 0.0001$].

The evaluation of amplitude in response to pleasant and neutral slides did not reveal any significantly difference between males and females at either F3 or F4 electrode sites.

Parietal sites. At site P3, women showed a P300 amplitude in response to unpleasant slides that was significantly higher compared to either pleasant [$F(1,62) = 14.491$; $^{*}p < 0.0001$] and neutral slides [$F(1,62) = 107.842$; $^{**}p < 0.001$]. Moreover, in women the P300 amplitude in response to unpleasant slides was significantly higher compared to men [$F(1,62) = 6.532$; $\bullet p < 0.013$].

In men, P300 amplitude in response to unpleasant slides was significantly higher at site P4 compared to P3 [$F(1,62) = 9.409$; $^{*}p < 0.003$], while in women it was significantly higher at site P3 compared to P4 [$F(1,62) = 7.867$; $\blacklozenge p < 0.012$].

The evaluation of amplitude in response to pleasant and neutral slides did not reveal any significant difference between males and females at either P3 or P4 electrode sites.

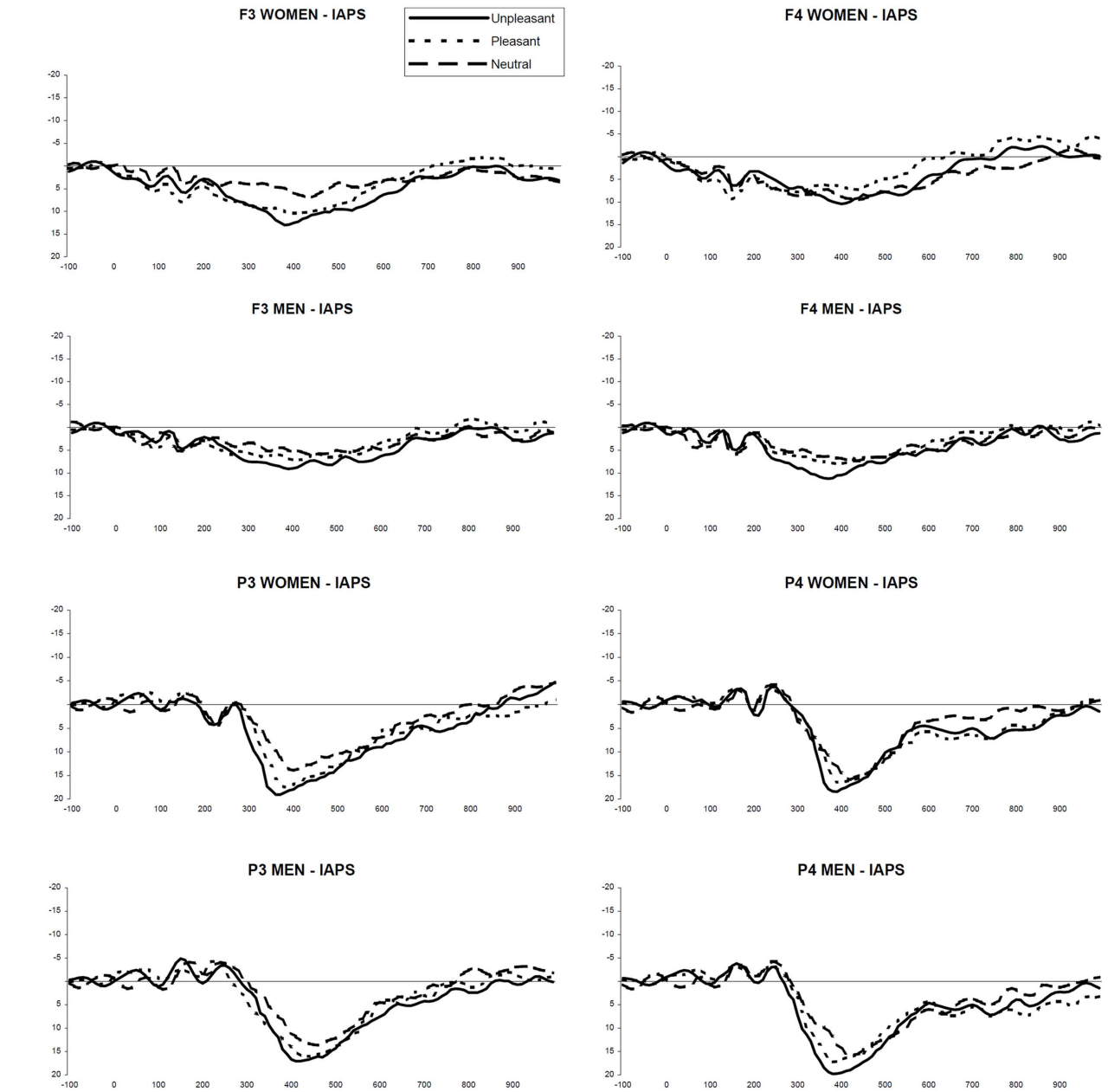

FIGURE 1 | Grand-average ERP waveforms in response to unpleasant, pleasant, and neutral IAPS stimuli, in women and men, at F3/F4 and P3/P4 electrode sites. Unbroken line: unpleasant slides; little dotted line: pleasant slides; regular dotted line: neutral slides.

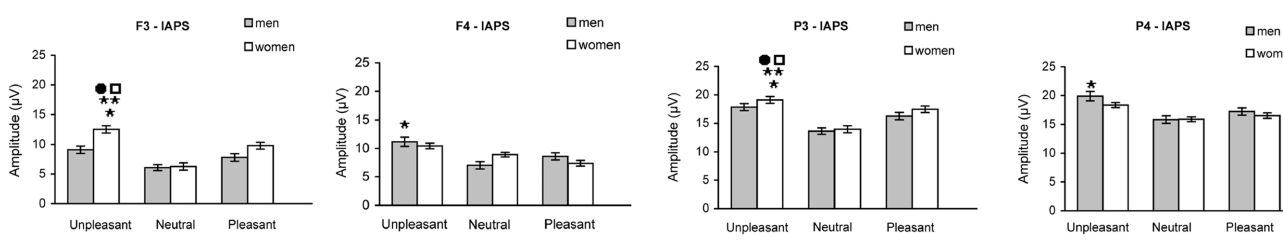

FIGURE 2 | Mean amplitude (±SEM) of P300 wave in men and women at F3/F4 and P3/P4 electrode sites, in response to unpleasant, pleasant, and neutral IAPS pictures.

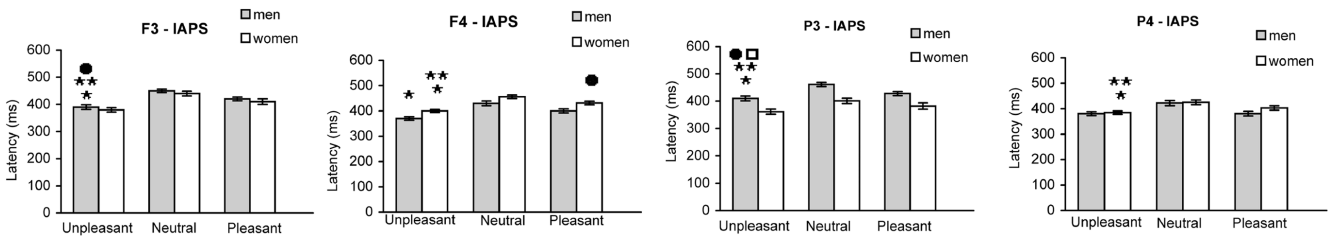

FIGURE 3 | Mean latency (±SEM) of P300 wave in men and women at F3/F4 and P3/P4 electrode sites, in response to unpleasant, pleasant, and neutral IAPS pictures.

P300 latency

The P300 latency was determined for P3/4, and F3/4 electrode sites in both men and women (**Figure 3**). All data were analyzed using ANOVA followed by Tuckey's test.

Frontal sites. At site F3, the P300 latency in women after the presentation of unpleasant slides was significantly shorter compared to both pleasant [$F(1,62) = 15.941$; $^{*}p < 0.0001$] and neutral slides [$F(1,62) = 84.071$; $^{**}p < 0.0001$]. Furthermore, latency at site F3 was significantly longer in men than in women [$F(1,62) = 3.982$; $\bullet p < 0.005$] in response to unpleasant slides. Indeed, the latency in response to unpleasant slides was significantly longer at F3 than at F4 electrode site in men [$F(1,62) = 11.108$; $^{*}p < 0.001$], but significantly longer at F4 than at F3 in women [$F(1,62) = 17.15$; $^{*}p < 0.01$].

At site F4 the P300 latency in response to the unpleasant slides was significantly longer in women compared to men [$F(1,62) = 25.710$; $^{**}p < 0.0001$]; moreover, the P300 latency in response to the pleasant slides was significantly longer in women compared to men [$F(1,62) = 6.160$; $\bullet p < 0.016$].

The evaluation of latency in response to pleasant and neutral slides did not reveal any significant difference between males and females at either F3 or F4 electrode sites.

Parietal sites. At site P3, in men, the P300 latency after the presentation of unpleasant slides was significantly shorter compared to both pleasant [$F(1,62) = 6.215$; $^{*}p < 0.015$] and neutral slides [$F(1,62) = 57.57$; $^{**}p < 0.0001$]. Furthermore, latency at site P3 was significantly longer in men than in women in response to unpleasant slides [$F(1,62) = 44.50$; $\bullet p < 0.001$]. Indeed, the latency in response to unpleasant slides was significantly longer at P3 than at P4 electrode site in men [$F(1,62) = 23.09$; $\blacklozenge p < 0.0001$], but significantly longer at P4 than at P3 in women [$F(1,62) = 9.54$; $^{*}p < 0.003$].

Finally, at site P4 the P300 latency in response to the unpleasant slides was significantly longer in women compared to men [$F(1,62) = 21.10$; $^{**}p < 0.003$].

The evaluation of latency in response to pleasant and neutral slides did not reveal any significant difference between males and females at either P3 or P4 electrode sites.

MEMORY

Free recall for the IAPS, evaluated on the basis of the number of pictures recalled, revealed that emotional content influenced memory both in men and women.

In fact, men recalled 7.34 (±1.78) unpleasant, 5.3 (±1.51) pleasant, and 2.45 (±1.52) neutral pictures; similarly, women recalled 8.3 (±2.16) unpleasant, 4.9 (±1.83) pleasant, and 1.87 (±1.28) neutral pictures.

The evaluation of memory for the IAPS slides showed that men recalled significantly more unpleasant slides compared to both pleasant ([$F(1,62) = 24.063$; $^{*}p < 0.0001$]) and neutral slides [$F(1,62) = 139.585$; $^{**}p < 0.0001$] (**Figure 4**); analogously, women recalled a significantly higher number of unpleasant than pleasant [$F(1,62) = 46.114$; $^{*}p < 0.001$] and neutral [$F(1,62) = 209.344$; $^{**}p < 0.0001$] slides.

The number of pleasant slides recalled by women and men was higher compared to neutral, even though this difference was statistically significant in men [$F(1,62) = 56.33$,$\nu p < 0.012$], but not in women [$F(1,62) = 2.59$, $p < 0.112$ n.s.; **Figure 4**].

The comparison between men and women revealed that females recalled a higher number of slides compared to males, even though the difference was statistically significant for the unpleasant [$F(1,62) = 8.62$, $p < 0.005$], but not for the pleasant [$F(1,62) = 0.93$, $p < 0.338$ n.s.] and neutral [$F(1,62) = 2.54$, $p < 0.116$ n.s.] slides.

TALKATIVENESS

In the free recall, the quantification of the number of words utilized by the subjects evidenced that women used 99.4 (±21.41) words to describe the unpleasant, 73.1 (±13.36) for the description of the pleasant, and 46.8 (±15.62) for the neutral slides. Our results show that female subjects utilized a statistically significant higher number of words to describe the unpleasant slides, compared to both pleasant [$F(1,62) = 35.53$; $\nu p < 0.01$] and neutral [$F(1,62) = 132.28$; $\nu\nu p < 0{,}01$] pictures; moreover, they used a statistically significant higher number of words to describe the pleasant compared to neutral slides [$F(1,62) = 56.33$; $^{***}p < 0{,}1$].

Men utilized 73.2 (±7.93) words to describe the unpleasant slides, 51.9 (±6.68) to describe the pleasant, and 46.5 (±15.44) to describe the neutral (**Figure 5**). Our findings reveal that male subjects utilized a statistically significant higher number of words to describe the unpleasant slides, compared to both pleasant [$F(1,62) = 147.62$; $\nu\nu p < 0.001$], and neutral [$F(1,62) = 78.68$; $\nu\nu\nu p < 0.01$]; moreover, they used a higher number of words to describe the pleasant compared to neutral [$F(1,62) = 2.59$; $p < 0.112$ n.s.], even though the difference is not statistically significant.

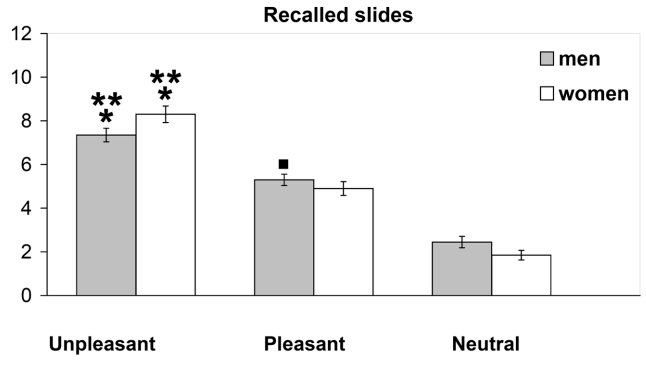

FIGURE 4 | Number of unpleasant, pleasant, and neutral IAPS pictures recalled by men and women.

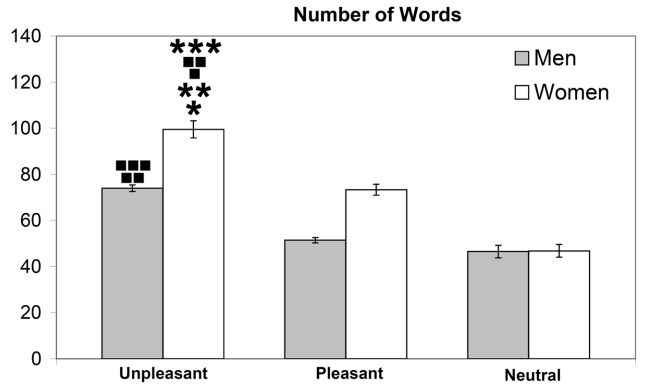

FIGURE 5 | Number of words utilized by men and women during the free recall for unpleasant, pleasant, and neutral IAPS pictures.

The comparative evaluation of the number of words utilized by men and women showed that women used a higher number of words compared to men to describe both the unpleasant [$F(1,62) = 40.98$;*$p < 0.001$], and pleasant pictures [$F(1,62) = 69.01$;**$p < 0.001$]; regarding the neutral slides, even though women used a higher number of words, compared to men, to describe them, the difference was not statistically significant [$F(1,62) = 0.011$; $p < 0.917$ n.s.].

DISCUSSION

Functional sex-related differences have been shown in brain correlates of emotional (Gasbarri et al., 2008b,c) and facial processing (Killgore and Yurgelun-Todd, 2001), working memory (Speck et al., 2000), auditory (Salmelin et al., 1999; Hulten et al., 2010) and language processing (Shaywitz et al., 1995; Pugh et al., 1996).

The present findings indicate that gender is a crucial variable in understanding cerebral hemisphere function, particularly in relation to emotional conditions, confirming that men and women differ in brain activation during cognitive tasks. In addition, we clearly confirm and extend our recent studies based on ERP, evaluating whether a sex-related hemispheric lateralization of electrical potentials elicited by emotional stimuli exists (Gasbarri et al., 2006, 2007, 2008a).

It is well known that P300 latency is considered a metric of stimulus classification speed, with shorter latencies thought to reflect superior cognitive performance (Duncan et al., 2009) and P300 latency and amplitude are negatively correlated (Polich, 2003).

As indexed by both amplitude and latency measures, the present results showing that emotional stimuli elicited a stronger P300 in women than in men, in the left hemisphere, and a stronger P300 in men than in women, in the right hemisphere, are in agreement with brain imaging studies indicating greater left hemisphere amygdala participation in processing memory of emotionally arousing material in women, but better right hemisphere amygdala processing into memory of the same material in men (Canli et al., 2002; Cahill, 2005, 2006, 2010; Andreano and Cahill, 2010).

Moreover, our data showing more robust right hemisphere P300 effects in men, and more robust left hemisphere P300 effects in women to arousing stimuli, reveal that the emotional slides were also better remembered, suggesting that the P300 effects could be related to the enhancement of memory.

In our study, the higher reactivity to unpleasant stimuli of women, compared to men, is in agreement with other research showing that females are more reactive to emotional unpleasant stimuli, particularly those that are threatening or traumatic (Whittle et al., 2011). Greater female reactivity has been found in the domains of self-report (Lang et al., 1993), behavioral response (Vigil, 2009), and physiological responding (Lithari et al., 2010), although some inconsistent findings were reported (Schienle et al., 2005; Fugate et al., 2009).

Many research works have revealed a gender-related dimorphism during the processing of emotional stimuli (Schirmer et al., 2002; Brebner, 2003; Campanella et al., 2004; Montagne et al., 2005; Scholten et al., 2005). Among these studies, one of the most noticeable observation is that men, compared to women, are less capable of labeling negative emotions (Li et al., 2008). Early reports showed that males are less accurate in recognizing emotions from faces, in particular in recognizing negative emotions such as fear, disgust, and sadness (Hall, 1978; Miura, 1993), and more recent studies, related to psychosocial aspects of depression, suggested that interpersonal communications through non-verbal emotional cues are more pronounced in women than in men (Harris, 2001; Campanella et al., 2004). Moreover, when asked to identify facial affects, healthy males had lower accuracy scores than healthy females in recognizing negative emotions, and the performances of male schizophrenic patients were impaired compared to female patients (Scholten et al., 2005). Similarly, women were more accurate in labeling negative emotions and identifying negative facial expressions, compared to men (Montagne et al., 2005). The advantage of women, compared to men, in identifying negative emotions, could be attributed to the reduced sensitivity to emotionally negative stimuli of men, compared to women, since it was shown that the same emotionally negative pictures activated more neural substrates as well as greater cerebral activation values (e.g., in amygdala) in females than in males (Wrase et al., 2003; Hofer et al., 2006; Li et al., 2008). These results evidence that women are more susceptible to negative emotions in life settings, which may be one important mechanism underlying their higher prevalence of affective disturbances, compared to men (Yuan et al., 2009).

It is widely recognized that the female brain has tremendous unique aptitudes outstanding verbal fluency, the ability to connect deeply in friendship, to read faces and tone of voice for emotions (Brizendine, 2006).

In the present study, the evaluation of the number of words utilized by male and female subjects which we counted during the free recall of emotional and neutral stimuli, showed that in both emotional and neutral pictures, women used a higher number of words compared to men. Sex-related differences in language processing and conversational behavior have long been an issue of scientific and also of public interest (Litosseliti, 2006; Mehl et al., 2007). Sexual dimorphism of cognitive ability has consistently been shown to occur in two domains: the former is represented by visuo-spatial ability (such as spatial perception, mental rotation, and mathematical tasks), and the latter includes tasks of verbal memory, verbal fluency, and speed of articulation. While men excel in visual–spatial ability skills, women outperform men in verbal fluency and episodic memory tasks, as well as in the use of prosodic information (Schirmer et al., 2002; Schirmer and Kotz, 2003; Bell et al., 2006).

The biological explanation for gender differences in language use hypothesizes that sex-related differences have resulted from evolutionary pressures for men to be more aggressive and self-assertive, and for women to be more nurturing and affiliative (Lippa, 2005; Luxen, 2005; Pellegrini and Archer, 2005; Anderson et al., 2006; Leaper and Ayres, 2007). A hypothesis underlying this view is that the brain of men and women tend to differ in its organization and functioning. Indeed, it was proposed that the female brain performs better in language ability (Andersen, 2006). This view is supported by studies indicating a slight advantage for girls over boys in language development and ability. There are also some studies suggesting average sex differences in brain lateralization (Gleason, 2002).

The cerebral substrate of the sex-related differences in cognition is actually still unclear. The most common hypothesis attributes sex-related differences in language processing to a left-hemispheric dominated activation in men, and a bilateral contribution of language-related brain areas in women (Josse and Tzourio-Mazoyer, 2004). Thus, males might predominantly use the left hemisphere for language functions, while women use both hemispheres. A more bilateral pattern of language representation could result in better verbal skills, while visuo-spatial processing would be inferior in subjects with bilateral language representation. Therefore, the female deficit in spatial performance is thought to arise from competition between verbal and spatial functions in the right hemisphere (Sommer et al., 2004). Many findings support this theory that sex differences in cognition result from more bilateral representation of language functions in women than in men. For example, it was showed that female stroke patients, compared to males, exhibited verbal impairment less frequently after lesions of the left hemisphere; in addition, a less pronounced asymmetry of the temporal planum in women, compared to men, was also reported (Foundas et al., 2002; Sommer et al., 2004).

Sex differences in conversational behavior and language processing have long been an issue of scientific and also public interest. Moreover, behavioral and functional imaging studies evidenced better performances of women, compared to men, in executive speech tasks (Weiss et al., 2006); in addition, differences in the influence of cognitive factors, such as speed of information processing, word knowledge, and long-term memory on verbal fluency factors were also reported (Weiss et al., 2006).

The popular stereotype that women are more talkativeness than men is often considered a scientific evidence (Brizendine, 2006), even though conflicting data still exist (Mehl et al., 2007), and many imaging studies failed to find such a generalized lateralization effect, reporting a left-lateralized activation in both sexes instead (Hill et al., 2006). This implies that the putative sex difference in language lateralization may be evidenced only with particular, as yet not defined, language tasks.

It is possible to hypothesized that part of the well known controversy about whether women speak more than men (Leaper and Ayres, 2007) may be related to the manner in which the speech is elicited, and the kind of task utilized (Wardle et al., 2011).

Most of the studies held gender constant, which might also contribute to different effects in same vs. opposite sex dyads. Future studies might also consider systematically varying the gender of the experimenter. Therefore, the hypothesis that differences in language lateralization underlie the general sex differences in cognitive performance has still to be confirmed, and the neuronal basis for these cognitive sex differences remains elusive.

Research into activation effects of ovarian hormones demonstrated that speech articulation and verbal fluency are enhanced at the high hormonal phases of the menstrual cycle (Wadnerkar et al., 2006). Many sex differences in language, that were once explained by testosterone-mediated effects alone, are now supposed to be the result of active differentiation in both sexes, i.e., feminisation in females and masculinisation in males (Whiteside et al., 2004). Ovarian estrogen influences the organization of many neurobehavioral systems in both non-humans and human species (Bimonte and Denenberg, 1999), and hormonal changes across the menstrual cycle affect several activational parameters at the neuroanatomical, neurofunctional, and behavioral levels (Sanders and Wenmoth, 1998; Rudick and Woolley, 2001; Sandstrom and Williams, 2001; Gasbarri et al., 2008c; Ossewaarde et al., 2010). Studies evaluating different tests of cognition and behavior, including reports of motor and perceptual skills and functional laterality in perception, showed that performance is systematically altered as a function of hormonal fluctuations during the menstrual cycle (Maki et al., 2002). Overall, the literature indicates that some abilities are positively, and others negatively influenced, during particular phases of the menstrual cycle corresponding to high levels of circulating estrogen (Hausmann et al., 2002) and, in some cases, also to progesterone (Whiteside et al., 2004).

However, much remains unclear about the organization and co-ordination of speech production systems as a function of activation by ovarian hormones across the menstrual cycle (Kitazawa and Kansaku, 2005).

The results of the current study support the view that sex differences in lateralization may not be a general feature of language processing but may be related to the specific condition, such as the emotional content of the stimuli used in the present research.

Moreover, taking into account that many data suggest that significantly different patterns of activation exist between men and women in different cognitive tasks and in various paradigms, the

present study reinforces the fact that gender matching is essential in functional studies, and supports the idea of exploring male and female populations as distinct groups.

In conclusion, according to previous studies indicating that men and women process emotional stimuli differently, our findings suggest the existence of gender-related neural responses to emotional stimuli, and highlight the neurobiological basis of mechanisms that may be underlying the gender disparity of neuropsychiatric diseases, such as mood disorders. In addition, considering that much remains unclear about the organization and co-ordination of speech production systems, as a function of activation by ovarian hormones across the menstrual cycle, future studies should take into account menstrual cycle stage and gonadal steroid hormone levels in the female subjects.

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Late protein synthesis-dependent phases in CTA long-term memory: BDNF requirement

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It has been proposed that long-term memory (LTM) persistence requires a late protein synthesis-dependent phase, even many hours after memory acquisition. Brain-derived neurotrophic factor (BDNF) is an essential protein synthesis product that has emerged as one of the most potent molecular mediators for long-term synaptic plasticity. Studies in the rat hippocampus have been shown that BDNF is capable to rescue the late-phase of long-term potentiation as well as the hippocampus-related LTM when protein synthesis was inhibited. Our previous studies on the insular cortex (IC), a region of the temporal cortex implicated in the acquisition and storage of conditioned taste aversion (CTA), have demonstrated that intracortical delivery of BDNF reverses the deficit in CTA memory caused by the inhibition of IC protein synthesis due to anisomycin administration during early acquisition. In this work, we first analyze whether CTA memory storage is protein synthesis-dependent in different time windows. We observed that CTA memory become sensible to protein synthesis inhibition 5 and 7 h after acquisition. Then, we explore the effect of BDNF delivery (2 μg/2 μl per side) in the IC during those late protein synthesis-dependent phases. Our results show that BDNF reverses the CTA memory deficit produced by protein synthesis inhibition in both phases. These findings support the notion that recurrent rounds of consolidation-like events take place in the neocortex for maintenance of CTA memory trace and that BDNF is an essential component of these processes.

Keywords: BDNF, memory persistence, protein synthesis, CTA, insular cortex

INTRODUCTION

Is now considered that memory can be divided into at least two stages: a protein synthesis-independent phase (short-term memory; STM), lasting minutes to hours and a protein synthesis-dependent phase (long-term memory; LTM), lasting several hours, days, or longer (Bailey et al., 2004; Medina et al., 2008). The process of developing stable memory is referred as consolidation. However, a single molecular cascade consisting of receptor activation, changes in protein phosphorylation, and a transient change in protein production, may be insufficient for the persistence of LTM (Wittenberg and Tsien, 2002; Tsien, 2006). Instead, it has been demonstrated the existence of a delayed protein synthesis-dependent phase for behavioral memory persistence, even several hours after memory acquisition (Grecksch and Matthies, 1980; Freeman et al., 1995; Quevedo et al., 1999; Igaz et al., 2002; Bekinschtein et al., 2007; Romero-Granados et al., 2010), showing that specific rounds of consolidation-like events take place for the maintenance of the memory trace.

Brain-derived neurotrophic factor (BDNF) has been considered as a protein synthesis product essential for the expression and persistence of long-term synaptic plasticity in the adult brain (Pang et al., 2004; Bekinschtein et al., 2008a,b; Moguel-Gonzalez et al., 2008). Alonso et al. (2002) showed that hippocampal BDNF is required for the formation of both short- and LTM and is continuously activated, in a time-dependent manner after consolidation for persistence of long-term hippocampal memory (Alonso et al., 2002). Previous studies in the rat hippocampus have demonstrated that application of BDNF is sufficient to rescue the late-phase of long-term potentiation (LTP) after protein synthesis inhibition (Pang et al., 2004). Recently, it has been demonstrated that 12 h after acquisition of a one-trial associative learning task, there is a novel protein synthesis and BDNF-dependent phase in the rat hippocampus that is critical for the persistence of LTM storage (Bekinschtein et al., 2007, 2008b). BDNF and its high affinity receptor tyrosine kinase receptor B (TrkB) are also abundantly expressed in the neocortex (Yan et al., 1997).

The insular cortex (IC) is a region of the temporal cortex in the rat that has been implicated in the acquisition and storage of different aversive motivated learning tasks like conditioned taste aversion (CTA). CTA is a very robust and widely used model for the study of learning and memory processes in which an animal acquires aversion to a novel taste when it is followed by digestive malaise (Bermudez-Rattoni, 2004; Bernstein and Koh, 2007; Bertrand et al., 2009). Our previous studies demonstrated that acute microinfusion of BDNF in anesthetized adult rats 24 h before CTA training enhances the retention of this task (Castillo et al., 2006). Moreover, it has been observed that protein translation inhibition impairs CTA consolidation but not acquisition (Rosenblum et al., 1993; Serova et al., 1996; Berman and Dudai, 2001; Moguel-Gonzalez et al., 2008) and we recently reported that acute

intracortical delivery of BDNF reverses the deficit in CTA memory, caused by inhibition of IC protein synthesis due to anisomycin administration (Moguel-Gonzalez et al., 2008). These evidences suggest that BDNF is an essential protein synthesis product for the establishment of the CTA–LTM. However, the requirement of protein synthesis and BDNF in different time windows after the acquisition of CTA has not been explored so far.

In the present study, we explore the protein synthesis-dependence of CTA–LTM in different time windows after acquisition. Then, we analyze the effect of BDNF delivery in the IC during those late protein synthesis-dependent phases.

MATERIALS AND METHODS

ANIMALS

A total of 104 male Wistar rats weighing 345–380 g were prepared for this experiment. Methods were carried out using adequate measures to minimize pain or discomfort, as outlined in the NIH Guide for the Care and Use of Laboratory Animals. They were housed individually under 12/12-h light-dark cycle, with food and water *ad libitum* (except where indicated) and an average room temperature of 22°C.

CANNULAE IMPLANTATION

Animals were implanted bilaterally with 23-gage stainless steel cannulae under anesthesia (Pentobarbital, 50 ml/kg i.p.) using a previously described procedure (Moguel-Gonzalez et al., 2008). The tips of the guide cannulae were aimed to 2 mm above the IC (Castillo et al., 2006). Microinjections were delivered through 30-gage dental needles as microinjectors that extended 2 mm below the previously implanted guide cannulae (reaching the IC area). Dental needle microinjectors were attached by polyethylene tubing to a 10-μl Hamilton syringe driven by a microinfusion pump (Cole Parmer Co.). After surgery animals were allowed to recover for 7 days. All groups were histologically analyzed in order to verify the injector tip location.

CTA TRAINING

After a 1-week recovery period, animals were introduced in the CTA training, as previously described (Moguel-Gonzalez et al., 2008; Rodriguez-Duran et al., 2011). Briefly, rats were deprived of water for 24 h and then habituated to drink water from a single graduated cylinder twice a day, during 10 min trials for 3 days. On the acquisition day, water was substituted for saccharin solution 0.1% (Sigma, St. Louis, MO, USA), and 10 min later, the animals received 7.5 ml/kg i.p. of a 0.15-M solution of LiCl, which induces digestive malaise. After three more days of baseline consumption, water was substituted newly by a 0.1% saccharin solution to test the aversion. The reduction of saccharin consumption with respect to baseline intake was used as a measure of strength of aversion.

EXPERIMENTAL DESIGN

It has been demonstrated that protein synthesis inhibition in the IC prevents CTA consolidation (Rosenblum et al., 1993; Berman and Dudai, 2001; Moguel-Gonzalez et al., 2008; Rodriguez-Duran et al., 2011). In order to explore the protein synthesis-dependence of CTA–LTM in different time windows after the acquisition session, intracortical microinfusions of anisomycin at a concentration that has been shown to act for 90 min with a 90% of protein synthesis inhibition (100 μg/μl per side, Sigma, St. Louis, MO, USA; Rosenblum et al., 1993; Moguel-Gonzalez et al., 2008) were applied at 3 (Ani3, $n = 10$), 5 (Ani5, $n = 8$), 7 (Ani7, $n = 10$), and 12 (Ani12, $n = 8$) hours after CTA acquisition in the IC (**Figure 1B**).

It has been observed that BDNF reverses the deficit in CTA memory caused by inhibition of IC protein synthesis due to anisomycin administration (Moguel-Gonzalez et al., 2008). In order to analyze if BDNF delivery is capable of revert the deficit in CTA memory during those time windows in which memory is sensitive to protein synthesis inhibition, intracortical microinfusions of BDNF (2 μg/2 μl per side, Alomone Labs, Jerusalem; Escobar et al., 2003; Castillo et al., 2006; Moguel-Gonzalez et al., 2008) were applied in the IC immediately after the anisomycin administration at 5 (AniBDNF5, $n = 8$) or 7 (AniBDNF7, $n = 9$) hours after CTA acquisition. In a similar manner, intracortical microinfusions of phosphate buffer solution (PBS) were applied immediately after the anisomycin administration at 5 (AniPBS5, $n = 10$) or 7 (AniPBS7, $n = 9$) hours after CTA acquisition, as BDNF vehicle. Artificial cerebrospinal fluid (ACSF, 1 μl/min) microinfusions were applied at 5 (ACSF5, $n = 12$) or 7 (ACSF7, $n = 10$) hours after CTA acquisition, as anisomycin vehicle. An additional group (CON, $n = 10$) was trained in CTA without any pharmacological treatment (**Figure 1C**). Upon completing the behavioral experiments, cannulated animals were histologically analyzed in order to verify the injector tip location.

RESULTS

HISTOLOGY

Histological examinations revealed that injectors were placed in the IC in all the groups (**Figure 1A**). Two animals with unclear cannulae placements were discarded; one of them was from the AniPBS7 group and the other from the CON group.

CTA BASELINE WATER CONSUMPTION

No significant differences were found among groups neither in the baseline water intake nor during the first presentation of the conditioned stimulus. The average baseline means (± SEM) of water intake were (in ml): 13.56 ± 0.050, 13.33 ± 0.31, 13.78 ± 0.49, $13. \pm 0.60$, 13.33 ± 0.46, 14.49 ± 0.60, 15.36 ± 0.34, 11.68 ± 0.72, 15.77 ± 0.39, 15.20 ± 0.51, and 15.98 ± 0.58 for the Ani3, Ani5, Ani7, Ani12, AniBDNF5, AniPBS5, ACSF5, AniBDNF7, AniPBS7, ACSF7, and CON groups, respectively.

CTA–LTM IS SENSITIVE TO PROTEIN SYNTHESIS INHIBITION AT 5 AND 7 H AFTER THE ACQUISITION OF THIS TASK

As shown in **Figure 2**, intracortical anisomycin microinfusions prevent the persistence of CTA memory when administered 5 and 7, but not 3 or 12 h after the acquisition session. ANOVA analysis for saccharin consumption during the aversion test revealed significant differences among groups ($F_{4,41} = 88.63$, $p < 0.001$). *Post hoc* analysis with Fisher's test showed that the Ani5 and Ani7 groups had a significant impairment in CTA memory when compared with Ani3, Ani12, and CON groups. The mean of saccharin consumption during the acquisition session for the Ani3, Ani5, Ani7, Ani12, and CON groups were 13.56 ± 1.05, 11.62 ± 3.00, 12.8 ± 1.87, 11.62 ± 1.63, and 15.46 ± 2.65, respectively. These results reveal that CTA–LTM becomes dependent on protein synthesis 5 and 7 h after memory acquisition.

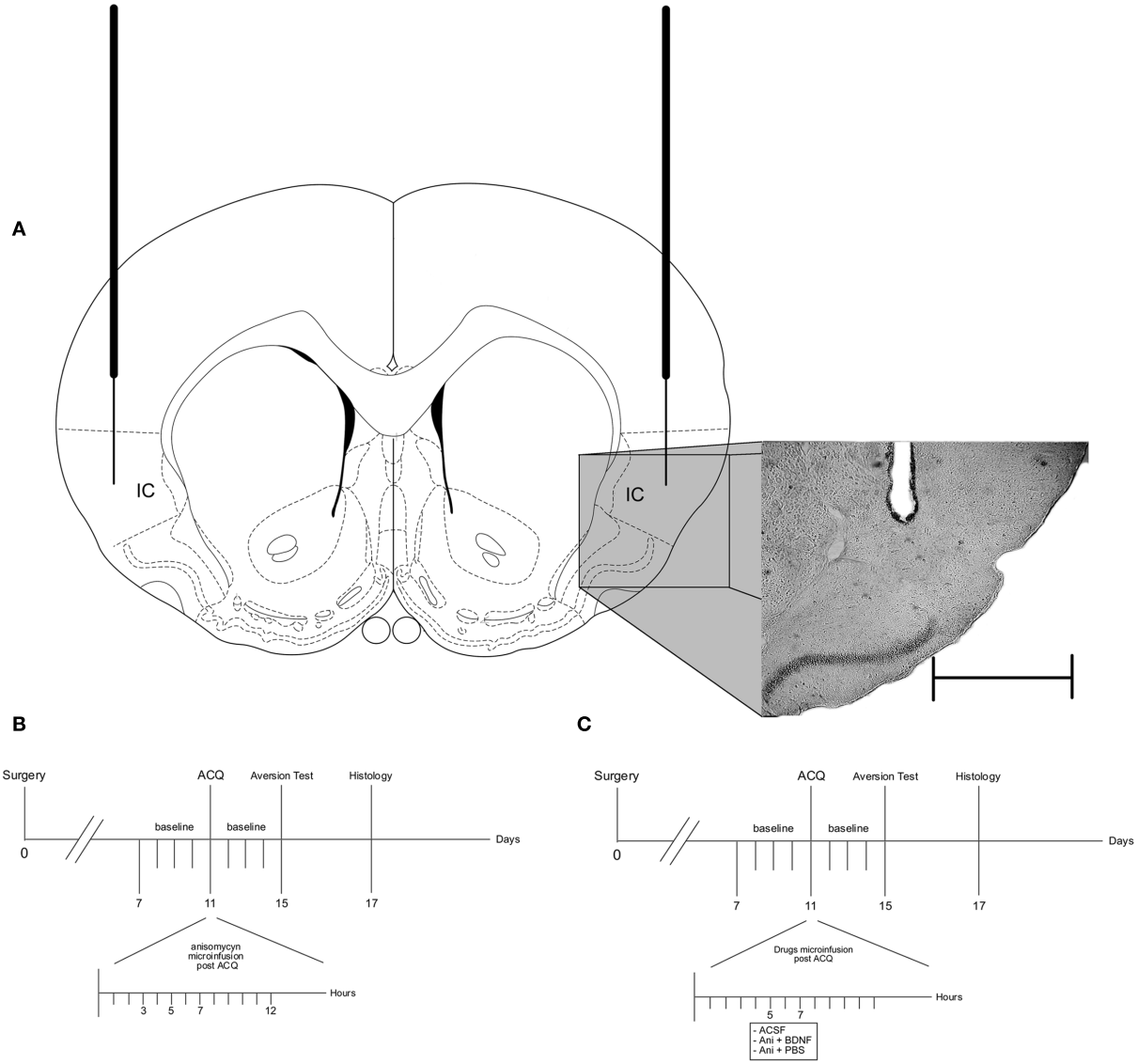

FIGURE 1 | Schematic representation of the experimental procedure. (A) Schematic diagram of the guide cannulae and microinjectors placement in a coronal plane. **(B)** Diagram of the experimental procedure for the analysis of late protein synthesis-dependent phases in CTA–LTM. **(C)** Diagram of the experimental procedure for the exploration of the BDNF effect on CTA deficit due to protein synthesis inhibition. Abbreviations: IC, insular cortex; ACQ, acquisition; ACSF, artificial cerebrospinal fluid; Ani, anisomycin; BDNF, Brain-derived neurotrophic factor; PBS, phosphate buffer solution. Scale bar: 1 mm.

BDNF REVERSES THE DEFICIT IN CTA–LTM CAUSED BY PROTEIN SYNTHESIS INHIBITION AT 5 AND 7 H AFTER THE ACQUISITION OF THIS TASK

Our results show that intracortical delivery of BDNF in the IC reverses the deficit in CTA memory caused by the inhibition of IC protein synthesis at 5 and 7 h after the acquisition of this task (**Figure 3**). ANOVA analysis for saccharin consumption during the aversion test revealed significant differences among those groups that were infused 5 h ($F_{3,36} = 87.39$, $p < 0.001$; **Figure 3**) as well as those groups that were infused 7 h after the acquisition session ($F_{3,34} = 102.09$, $p < 0.001$, **Figure 3**). *Post hoc* analysis with Fisher's test showed that AniBDNF5 and AniBDNF7 groups had a significant improvement in CTA memory persistence as indicated by the decrease of saccharin consumption when compared with the AniPBS5 and AniPBS7 groups. No significant differences were observed among the AniBDNF5, AniBDNF7, ACSF5, ACSF7, and CON groups. The mean of saccharin consumption during the acquisition session for the AniBDNF5, AniPBS5, ACSF5, AniBDNF7, AniPBS7, and ACSF7 groups were 11.55 ± 1.33, 13.00 ± 1.49, 13.83 ± 1.27, 10.88 ± 1.83, 14.22 ± 1.39, and 15.20 ± 1.62, respectively. These results demonstrate that intracortical delivery of BDNF reverses the deficit in CTA memory maintenance caused by inhibition of IC protein synthesis even 5 and 7 h after memory acquisition.

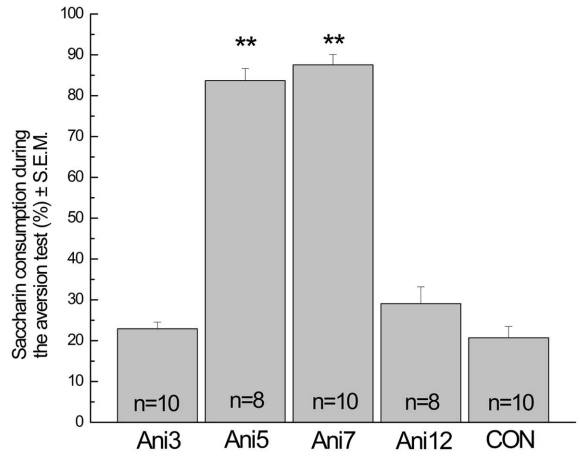

FIGURE 2 | Conditioned taste aversion-LTM is sensitive to protein synthesis inhibition at 5 and 7 h after the acquisition of this task. Acute intracortical microinfusion of anisomycin (100 μg per side) carried out 5 (Ani5) or 7 (Ani7) but not 3 (Ani3) or 12 (Ani12) hours after memory acquisition impairs CTA memory. An additional control group (CON) remains intact during the whole procedure. $^{**}p < 0.001$.

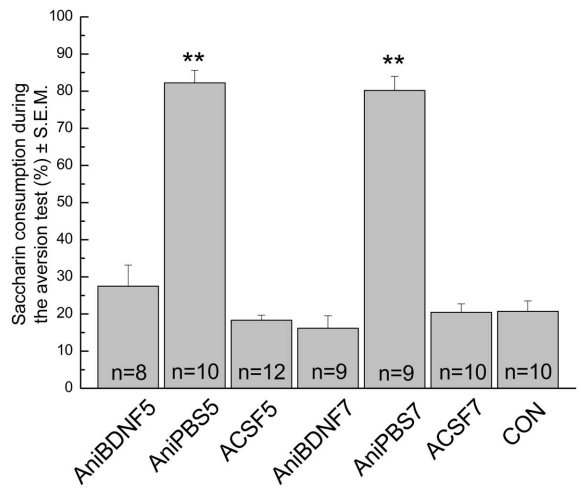

FIGURE 3 | Brain-derived neurotrophic factor (BDNF) reverses the deficit in CTA–LTM caused by protein synthesis inhibition at 5 and 7 h after the acquisition of this task. Acute intracortical microinfusion of BDNF (2 μg per side) immediately after anisomycin administration (100 μg per side) reverses the impairment in CTA memory. Animals received intracortical infusion of artificial cerebrospinal fluid (ACSF5 and ACSF7), anisomycin plus BDNF (AniBDNF5 and AniBDNF7) or anisomycin plus phosphate buffer solution (AniPBS5 and AniPBS7). An additional control group (CON) remains intact during the whole procedure. $^{**}p < 0.001$.

DISCUSSION

A great body of evidence shows that LTM trace is sensitive to protein synthesis inhibition during the first hours after memory acquisition (McGaugh, 1966, 2000; Davis and Squire, 1984; Kandel, 2001; Barrientos et al., 2002; Dudai, 2002). However, it has been proposed that a single molecular cascade triggered during learning might not be sufficient to account for the persistence of memory in the mammalian brain (Wittenberg and Tsien, 2002), raising the notion that recurrent rounds of consolidation-like events are required for the persistence of LTM in the hippocampus (Bekinschtein et al., 2007). Several studies have been demonstrated that protein synthesis is also critical for memory persistence at least in two time periods: around the time of and several hours after training (Quevedo et al., 1999; Igaz et al., 2002; Bekinschtein et al., 2008b; Romero-Granados et al., 2010). These late protein synthesis-dependent periods have been reported in the rat hippocampus 4 h after recognition memory formation (Romero-Granados et al., 2010) as well as 12 h after acquisition of a one-trial IA task (Bekinschtein et al., 2007, 2008b).

The present results show that the persistence of the CTA memory requires a late protein synthesis-dependent phase 5 and 7 h but not 3 or 12 h after memory acquisition in the IC, a neocortical area where certain forms of aversive memory are likely to reside (Bermudez-Rattoni, 2004; Moguel-Gonzalez et al., 2008). These results suggest that the reactivation of a cascade of molecular and cellular events takes place in the IC for the maintenance of CTA memory trace.

Brain-derived neurotrophic factor is now considered as an essential protein synthesis product for the establishment of synaptic plasticity (Minichiello, 2009; Bekinschtein et al., 2010; Musumeci and Minichiello, 2011). Our previous studies show that acute intrahippocampal microinfusion of BDNF induces LTP in the hippocampal DG-CA3 (mossy fiber) projection. This potentiation was accompanied by a presynaptic structural long-lasting reorganization and a change in the possibility to induce subsequent LTP at the mossy fiber pathway (Gomez-Palacio-Schjetnan and Escobar, 2008; Schjetnan and Escobar, 2010). In addition, it has been shown that BDNF induces long-term modifications that underlie the late-phase of LTP in the CA1 area of the hippocampus (Pang et al., 2004). Moreover, intrahippocampal microinfusion of BDNF induces memory persistence by itself transforming a non-lasting LTM trace into a persistent one (Bekinschtein et al., 2008b). In the other hand, our studies in the IC have shown that exogenous application of BDNF in the IC enhances synaptic efficacy in the basolateral amygdaloid nucleus–IC projection and modifies the retention of CTA (Escobar et al., 2003; Castillo et al., 2006; Castillo and Escobar, 2011) and recently we showed that the deficit in CTA memory caused by the inhibition of protein synthesis in the IC, during the acquisition session is reversed by an acute intracortical delivery of BDNF (Moguel-Gonzalez et al., 2008).

In the present work we show that acute intracortical delivery of BDNF reverses the deficit in CTA memory caused by the inhibition of IC protein synthesis due to anisomycin administration 5 and 7 h after memory acquisition. BDNF is essential for the consolidation of CTA memory early after acquisition (Moguel-Gonzalez et al., 2008) and the present data reveals that this neurotrophin is also required for CTA–LTM persistence even several hours after the acquisition session.

In the same order of ideas, it has been demonstrated that BDNF endogenous expression is modulated in different time windows during the formation of LTM (Ou et al., 2010; Ma et al., 2011). In a recent study Ou et al. (2010) showed that fear conditioning

training triggers two peaks of BDNF expression in the amygdala, at 1 and 12 h after conditioning. In the hippocampus, IA memory presents a BDNF-dependent phase 12 h after training and BDNF infusion into this time window transform a non-lasting LTM trace into a persistent one (Bekinschtein et al., 2008b). These evidences suggest that an endogenous increase in BDNF production is necessary for the maintenance of LTM. In a similar manner, a recent study of Ma et al. (2011) shows the specific regional involvement of BDNF secretion and synthesis in the CTA–LTM. They reported that the CTA training induces an increase in BDNF levels at the IC. This increase starts at 2 h, peaked at 6 and returned to baseline at 8 h after CTA acquisition (Ma et al., 2011). Accordingly, in the present work, we observed that intracortical delivery of BDNF is sufficient to reverse the LTM impairment produced by anisomycin microinfusion, within the time of BDNF expression presented by Ma et al., 2011; 5 and 7 h after the CTA acquisition), suggesting that a late-phase of endogenous release of BDNF is required for the maintenance of CTA–LTM.

Release of BDNF initiates a cascade of molecular mechanisms related with activity-dependent synaptic changes (Minichiello, 2009; Musumeci and Minichiello, 2011). This neurotrophin and its high affinity receptor TrkB trigger a molecular cascade of proteins, as the mitogen-activated protein kinase (MAPK), the phospholipase C-gamma (PLC-gamma) and the phosphatidylinositol-3 kinase (PI-3K) pathways (Minichiello et al., 2002). Through these pathways, BDNF regulates the activity-dependent changes in synaptic properties related with the maintenance of the LTM of several learning tasks (Yamada and Nabeshima, 2003). For example, training in the IA task requires a late BDNF-dependent phase in the hippocampus 12 h after training (Bekinschtein et al., 2007). In addition, it has been observed a late increase in extracellular signal-regulated kinases (ERK) phosphorylation levels in the hippocampus 12 h after IA training, and intrahippocampal infusion of the MAPK kinase (MEK) inhibitor, U0126, resulted in a deficit in LTM retention (Bekinschtein et al., 2008b). Our previous studies on the IC showed that microinfusion of BDNF previous to CTA training modifies the retention of this task, through the activation of MAPK and PI-3K at the IC (Castillo et al., 2006; Castillo and Escobar, 2011). The mentioned molecular cascades converge in the activation of transcription factors, such as cyclic adenosine monophosphate response element-binding protein (CREB), which induce gene expression (e.g., Arc and Zif 286) and thereby LTM-related synaptic changes (Minichiello et al., 2002; Minichiello, 2009).

Although the molecular mechanisms triggered by BDNF underlying the persistence of behavioral memories remains unclear, it has been observed that BDNF enhances the expression of the protein kinase M ζ (PKMζ), an atypical isoform of the protein kinases C (PKC) which plays a crucial role in the persistence of LTM in several behavioral tasks, including CTA (Shema et al., 2007, 2009, 2011; Gamiz and Gallo, 2011; Sacktor, 2011). The local synthesis of BDNF and PKMζ are required for the maintenance of certain forms of LTP (Sajikumar and Korte, 2011) and recently, Mei et al. (2011) demonstrated that BDNF is capable to sustain the late-phase of LTP through PKMζ in a protein synthesis-independent manner, providing a strong mechanistic link between BDNF and PKMζ in the persistence of synaptic plasticity.

In summary, the present results show that CTA memory becomes sensible to protein synthesis inhibition 5 and 7 h after memory acquisition, revealing that new protein synthesis is required for the maintenance of CTA–LTM even hours after acquisition. Furthermore, BDNF reverses the CTA memory deficit produced by protein synthesis inhibition during both late-phases, showing that BDNF is an essential protein product for the persistence of CTA in different post-acquisition time windows. These evidences suggest that a new round of consolidation-like events take place in the IC for the maintenance of the CTA memory trace and stress the role of BDNF as a key protein which promotes the persistence of LTM in the neocortex.

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Striatal intrinsic reinforcement signals during recognition memory: relationship to response bias and dysregulation in schizophrenia

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Ventral striatum (VS) is a critical brain region for reinforcement learning and motivation, and VS hypofunction is implicated in psychiatric disorders including schizophrenia. Providing rewards or performance feedback has been shown to activate VS. Intrinsically motivated subjects performing challenging cognitive tasks are likely to engage reinforcement circuitry even in the absence of external feedback or incentives. However, such intrinsic reinforcement responses have received little attention, have not been examined in relation to behavioral performance, and have not been evaluated for impairment in neuropsychiatric disorders such as schizophrenia. Here we used fMRI to examine a challenging "old" vs. "new" visual recognition task in healthy subjects and patients with schizophrenia. Targets were unique fractal stimuli previously presented as salient distractors in a visual oddball task, producing incidental memory encoding. Based on the prediction error theory of reinforcement learning, we hypothesized that correct target recognition would activate VS in controls, and that this activation would be greater in subjects with lower expectation of responding correctly as indexed by a more conservative response bias. We also predicted these effects would be reduced in patients with schizophrenia. Consistent with these predictions, controls activated VS and other reinforcement processing regions during correct recognition, with greater VS activation in those with a more conservative response bias. Patients did not show either effect, with significant group differences suggesting hyporesponsivity in patients to internally generated feedback. These findings highlight the importance of accounting for intrinsic motivation and reward when studying cognitive tasks, and add to growing evidence of reward circuit dysfunction in schizophrenia that may impact cognition and function.

Keywords: memory, reward, intrinsic motivation, ventral striatum, schizophrenia

INTRODUCTION

Any account of cognition is incomplete without integrating the influence of emotion and motivation. Interactions between cognition, emotion, and motivation have attracted increasing research, both in healthy individuals and patients with psychiatric disorders such as schizophrenia (Barch, 2005; Phelps, 2006; Satterthwaite et al., 2009, 2010; Duckworth et al., 2011; Murty et al., 2011).

Building upon an extensive animal literature, human fMRI research highlights the role of the ventral striatum (VS) in driving both motivation and learning in response to a wide range of rewards (Robbins and Everitt, 1996; McClure et al., 2004). While most fMRI studies focusing on VS have examined responses to explicit delivery of reinforcers such as money, VS activation has also been reported in response to purely cognitive feedback such as information reflecting performance accuracy (Rodriguez et al., 2006; Tricomi and Fiez, 2008). Rather than reflecting reward value *per se*, VS fMRI responses have been shown to reflect positive prediction errors that occur when outcomes are better than expected (McClure et al., 2003; Schultz, 2010). VS thus may be a critical hub for motivation–cognition interactions, especially when cognitive performance can generate positive outcomes.

Memory is a critical aspect of cognition that is strongly modulated by inter-related emotional and motivational systems (Dere et al., 2010; Murty et al., 2011). Human fMRI studies have demonstrated enhancement of memory encoding by rewards that increase dopamine signaling and VS responses (Adcock et al., 2006; Wittmann et al., 2008; Bunzeck et al., 2011). Less attention has been paid to the role of reward and reinforcement in memory retrieval. However, successful retrieval is likely to itself produce reward signals as task success is psychologically rewarding. fMRI activation in VS/anterior caudate in response to successful retrieval has been observed in some studies including two meta-analyses

(von Zerssen et al., 2001; Iidaka et al., 2006; McDermott et al., 2009; Spaniol et al., 2009), but has generally not been interpreted in terms of reward processes given the cognitive focus of the research (see von Zerssen et al., 2001 for an exception). A single fMRI study has explicitly focused on striatal responses as a reflection of goal-directed reward processing during memory retrieval (Han et al., 2010). In that study, anterior caudate robustly activated to correctly recognized targets even in the absence of external rewards or trial-by-trial feedback. By manipulating task rewards, Han et al. (2010) further demonstrated that striatal activation during successful recognition was not due to memory retrieval *per se*, but to the reward significance of correct performance.

Schizophrenia is associated with a range of cognitive deficits, including prominent recognition memory deficits (Saykin et al., 1991; Heinrichs and Zakzanis, 1998; Ragland et al., 2004; Gur et al., 2007). Deficits in motivation are also often present (Wolf, 2006; Foussias and Remington, 2008). Hypofunction of VS has been found in schizophrenia in response to primary and monetary rewards as well as novelty (Juckel et al., 2006; Wolf et al., 2008; Waltz et al., 2009, 2010). VS hypofunction has also been observed in patients with schizophrenia during a cognitive inhibitory control task (Vink et al., 2006), and in dorsal striatum in a working memory task (Koch et al., 2008). However, striatum has not been a regional focus of interest in most recognition memory studies in schizophrenia. VS deficits in this context have not to our knowledge been reported, and are not identified in recent meta-analyses (Achim and Lepage, 2005; Ragland et al., 2009).

Here we examine data from controls and patients with schizophrenia performing a challenging old vs. new visual recognition memory task, focusing on VS responses during successful target recognition. Our hypothesis was that intrinsically motivated performance would generate VS reward prediction error signals in controls, an effect diminished in patients. Consistent with Han et al. (2010) and with the intuitive notion that being correct is intrinsically rewarding, we expected VS activation to successful retrieval even in the absence of explicit feedback or external rewards, as subjects internally evaluated their own performance relative to their expectations. We hypothesized that a difficult memory task would produce lower expectations of correct retrieval and hence generate robust prediction errors and VS activation for correctly identified targets. A previously untested prediction of this hypothesis is that subjects with a more conservative response bias, and hence a lower tendency to identify items as previously seen, would generate larger prediction errors manifesting as greater VS activation to correct retrieval. Given the association of schizophrenia with deficits in striatal function as well as impairments in memory, reward processing, and motivation, we predicted patients would show reduced activation of VS during successful retrieval, and a lack of VS modulation by response bias.

MATERIALS AND METHODS

PARTICIPANTS

The study included 26 clinically stable patients with schizophrenia (12 female), and 27 healthy comparison subjects. Two control subjects were excluded due to excessive motion, leaving a final sample of 25 healthy comparison subjects (12 female). The groups did not differ significantly with respect to age, sex, handedness, education level, or parental education level (**Table 1**).

All subjects were evaluated with a structured clinical interview (First et al., 2001) and symptom scales including SANS and SAPS (Andreasen, 1984a,b) by individuals trained to a criterion reliability of ICC = 0.90. Collateral medical and psychiatric histories were obtained from records, family members, and caregivers. Patients met criteria for a diagnosis of DSM-IV schizophrenia as established in a consensus conference based on all information obtained during study procedures. Except for one unmedicated individual, patients were on a stable antipsychotic regimen (4 on first-generation, 21 on second/third-generation). No participants were diagnosed with any other psychiatric disorders, recent substance use disorders (within past 6 months) or current use other than nicotine, or any other disease affecting brain function. All participants were volunteers at the Schizophrenia Research Center at the Hospital of the University of Pennsylvania and provided written consent after receiving a full description of study procedures. Study procedures were approved by the University of Pennsylvania IRB.

TASK

Participants were presented with a series of 60 images of fractals in a forced choice recognition memory paradigm (**Figure 1**). Half of the fractals had been previously displayed once each for 2 s as salient distractors during a visual oddball task performed ∼10 min earlier in the scanning session, yielding 30 targets and

Table 1 | Demographics, clinical variables, and performance.

	Patients ($n = 26$)	Controls ($n = 25$)	p Value
Gender (% female)	46	48	0.99
Handedness (% right)	85	92	0.67
Age (year)	38.0 (10.7)	38.0 (10.3)	0.99
Education (year)	13.1 (2.6)	14.3 (1.9)	0.06
Parental education	13.31 (2.75)	13.69 (3.18)	0.63
SANS global	1.53 (0.82)		
SAPS global	1.40 (0.99)		
Overall accuracy	0.57 (0.09)	0.65 (0.07)	0.002
Hit rate	0.46 (0.20)	0.50 (0.14)	0.38
Miss rate	0.54 (0.21)	0.50 (0.14)	0.38
Correct rejection rate	0.69 (0.21)	0.80 (0.15)	0.049
False alarm rate	0.31 (0.21)	0.20 (0.15)	0.049
P_r	0.15 (0.17)	0.29 (0.13)	0.002
B_r	−0.13 (0.21)	−0.21 (0.17)	0.23
Hit RT (s)	1.17 (0.21)	1.07 (0.20)	0.11

Values are mean (SD) except where otherwise noted. p Values are two-tailed based on unpaired t-tests (except for gender and handedness which used Fisher's exact test). SAPS and SANS scores are the average of the global items (SANS attention subscale not included). Proportions for hits, misses, correct rejections, and false alarms are presented here as raw rates without the addition of 0.5 to the numerator and 1 to the denominator utilized by Snodgrass and Corwin (1988; see formulas in Materials and Methods).

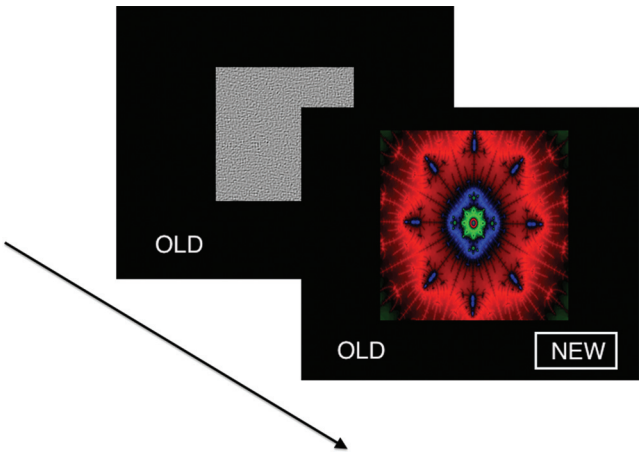

FIGURE 1 | Visual recognition task. In each of 60 trials, a unique fractal image was presented in a forced choice recognition memory paradigm. Half of the fractals had been previously displayed once each as salient distractors during a visual oddball task performed ∼10 min earlier, yielding 30 targets and 30 foils. Stimuli were presented for 3 s in a fast event-related design, with a variable 0–18 s interstimulus interval during which a homogeneous visual noise gray background was displayed.

30 foils. Subjects were not specifically instructed to remember these items. The oddball task was adapted from an experiment previously described in detail (Gur et al., 2007). In the present recognition memory experiment, each stimulus was presented for 3 s in a fast event-related design, with a variable 0–18 s (mean 3.4 s) interstimulus interval during which a homogeneous visual noise gray background was displayed. Responses and reaction times were recorded with a two-button response pad (FORP^{TM}, Current Design Inc., Philadelphia, PA, USA). Total task duration was 6.3 min.

IMAGE ACQUISITION

Data were acquired on a 3-Tesla Siemens Magnetom TIM TRIO system (Erlangen, Germany) using an 8-channel head coil. A TI weighted whole-brain structural scan was acquired for use in co-registration to a standard brain atlas (MPRAGE, TR = 1630 ms, TE = 3.87 ms, FOV = 250 mm, matrix = 192 × 256, effective voxel resolution of 1 mm × 1 mm × 1 mm). Functional BOLD images were acquired using a 2-D echo-planar sequence (TR = 3000 ms, TE = 30 ms, FOV = 192, slice thickness = 3 mm, matrix = 64 × 64, effective resolution = 3 mm × 3 mm × 3 mm).

PERFORMANCE ANALYSIS

Subject performance during recognition trials was evaluated using a measure of discrimination index, P_r, and a measure of response bias, B_r (Snodgrass and Corwin, 1988). These variables were computed with the following formulas:

$$\begin{aligned}\text{Hit rate (HR)} &= (0.5 + \text{number correct targets})/\\ &\quad (1 + \text{number total targets})\\ \text{False alarm rate (FR)} &= (0.5 + \text{number incorrect foils})/\\ &\quad (1 + \text{number total foils})\\ P_r &= \text{HR} - \text{FR}\\ B_r &= [\text{FR}/(1 - P_r)] - 0.5\end{aligned}$$

P_r provides a measure of discrimination accuracy that is unbiased by a subject's tendency toward a greater total number of responses; higher values reflect a greater degree of accuracy in discriminating between old and new items. B_r measures the overall tendency of a subject to respond that any given stimulus is new or old. Following Sergerie et al. (2007), we adapted the original B_r formula (where B_r $r = 0.5$ reflected neutrality or no bias), subtracting 0.5 so that 0 becomes the neutral point. Negative B_r values thus denote a tendency to respond that previously displayed images are new (conservative response bias), whereas positive B_r values indicate a tendency to call new stimuli old (liberal response bias). P_r, B_r and response times (RT, within-subject median for HITS) were compared between groups using unpaired t-tests ($\alpha = 0.05$, two-tailed).

IMAGE ANALYSIS

Images were preprocessed and analyzed using FEAT, part of FSL: FMRIB's Software Library (www.fmrib.ox.ac.uk/fsl). Images were slice-timing corrected, motion corrected to median image using a tri-linear interpolation with 6 degrees of freedom, high-pass filtered (100 s), spatially smoothed using a Gaussian kernel of 6 mm FWHM, and grand mean scaled. Subject-level time-series analysis was carried out using a general linear model with four event types: target correct (HIT), target incorrect (MISS), foil correct (correct rejection, CR), and foil incorrect (false alarm, FA). All event types were convolved with double-gamma canonical hemodynamic response functions. Six motion parameters were included in the model as confound regressors. Group level mixed-effects analyses (FLAME 1) were performed for controls, patients, controls > patients, and patients > controls.

Given our *a priori* hypotheses regarding reward signals in the VS and the low sensitivity of whole-brain analysis for small subcortical structures, we performed a region of interest (ROI) analysis of the HIT > baseline contrast within bilateral VS, defined as the combination of 14 mm radius spheres centered on MNI coordinates ±11, 9, −2 from Knutson et al. (2005). In order to evaluate effects outside of the VS, we followed this ROI analysis with an exploratory whole-brain analysis of the HIT > baseline contrast. We focused on the HIT > baseline contrast because it related most clearly to our hypotheses regarding successful task performance; the results of Han et al. (2010) suggested that correct target identification is more goal-relevant to subjects than foil performance when no explicit incentives are provided. In order to evaluate the specificity of the HIT > baseline response with a tighter contrast, we additionally evaluated the contrast of HIT > MISS on a voxelwise basis. We observed group differences in the number of correct and incorrect foil responses but not the number of HIT or MISS responses, adding an important *post hoc* rationale to our *a priori* hypothesis-driven decision to focus on the above contrasts. To investigate the relationship between task performance and brain responses, B_r, and P_r were included as covariates in both ROI and whole-brain analyses. Both whole-brain and ROI analyses were corrected for multiple comparisons using a Monte Carlo simulation with AFNI AlphaSim (R. W. Cox, National Institutes of Health) with 10,000 permutations and a probability of spatial extent $p < 0.01$. For the VS ROI, mask volume was 22,328 mm^3, and the whole-brain mask volume was 1,415,672 mm^3. Voxel height threshold for all analyses

was $Z > 2.33$ except for the exploratory whole-brain HIT > baseline analysis which used $Z > 3.10$ in order to separate anatomically meaningful clusters for this robust effect.

RESULTS

BEHAVIORAL RESULTS

Patients performed significantly worse than controls in overall accuracy and discrimination accuracy (P_r), but HIT rate and HIT reaction times did not significantly differ (**Table 1**). The reduction in discrimination accuracy in patients related to decreased foil performance with an increase in FAs. Both groups showed a conservative response bias as indicated by a negative B_r which did not differ significantly between groups. In both groups, a more negative (conservative) B_r correlated with lower hit rate (proportion of target *trials* which were hits; control $r = 0.75$, patient $r = 0.79$), a relationship implicit in the formula for calculating B_r. However, more negative B_r also correlated with *higher target response specificity* (proportion of target *responses* which were hits; control $r = -0.81$, patient $r = -0.57$). A *post hoc* simulation analysis (performed in MATLAB, code available upon request) demonstrated that this relationship was statistically significant (control, $p = 0.0001$; patient, $p = 0.01$) and did not result from a trivial mathematical relationship: the correlation observed across many "subjects" with random performance was essentially 0 ($r = -0.03$).

A PRIORI ROI RESULTS

Our *a priori* analysis of HIT > baseline in the VS revealed a robust response among healthy subjects in bilateral regions encompassing the anterior caudate, nucleus accumbens, and putamen (**Figure 2**). In contrast, patients did not show significant HIT > baseline activation in this region. Accordingly, the between-group contrast revealed greater activation for controls than patients in VS (significant on left, subthreshold on right).

WHOLE-BRAIN ANALYSIS

As expected, healthy subjects robustly activated regions associated with motivation and reinforcement processing in the HIT > baseline contrast, including the VS, midbrain, anterior insula/posterior orbital frontal cortex, and dorsal anterior cingulate/paracingulate (**Figure 3**; **Table 2**). Patients activated a qualitatively similar set of regions. The voxelwise contrast of controls > patients contrast

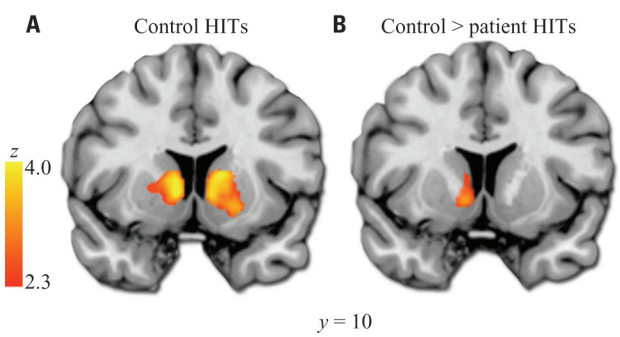

FIGURE 2 | Ventral striatum HIT > baseline ROI analysis. (A) Results in healthy controls. **(B)** Results from control > patient contrast. Images thresholded at $Z > 2.3$ and cluster corrected for multiple comparisons with spatial extent $p < 0.01$. Note: A similar cluster to that shown in **(B)** was the only significant result of the exploratory whole-brain controls > patients analysis. Left hemisphere shown on left side of images.

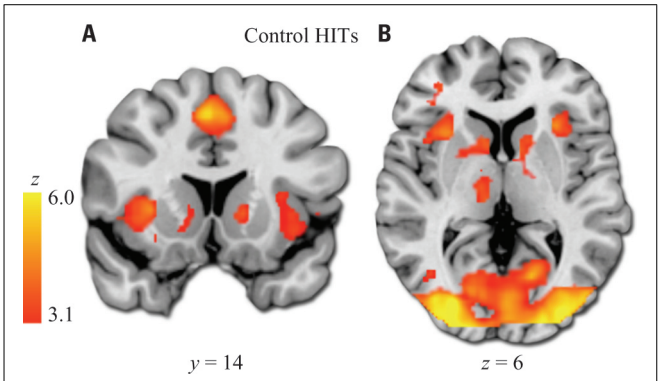

FIGURE 3 | HIT > baseline whole-brain exploratory voxelwise analysis. Coronal **(A)** and transverse **(B)** images showing activation of the reward and motivation network regions including anterior insula, dACC, and striatum. Images thresholded at $Z > 3.1$ and cluster corrected for multiple comparisons with spatial extent $p < 0.01$.

Table 2 | HIT > baseline whole-brain significant clusters.

Voxels	Region	Max-*Z*	*x*	*y*	*z*
Controls					
24184	Visual cortex/fusiform/ cerebellum/midbrain*/ sup. parietal	7.55	28	−56	−20
1580	R insula/OFC/MFG	5.48	32	26	−2
1137	L insula/OFC/MFG	5.58	−32	20	−2
938	dACC/paracingulate	5.62	−4	14	48
862	L IFG	5.11	−44	4	30
483	R IFG	5.26	38	2	30
480	R caudate/thalamus	4.18	14	−12	10
327	L caudate	4.21	−10	8	0
239	L thalamus	4.54	−12	−16	8
Patients					
16629	Visual cortex/fusiform/ cerebellum	7.28	22	−92	6
4304	Superior parietal	5.64	−50	−24	50
610	dACC/paracingulate	4.8	0	6	48
292	L insula	4.64	−34	16	4
257	L thalamus	4.29	−14	−18	8
250	R insula	4.67	34	18	4
Controls > patients					
45	L ventral striatum	3.45	−14	0	−8

Data thresholded at $Z > 3.1$ and corrected for multiple comparisons using AlphaSim with a spatial extent $p < 0.01$.
OFC, orbitofrontal cortex; MFG, middle frontal gyrus; IFG, inferior frontal gyrus; dACC, dorsal anterior cingulate cortex. Peak-Z x, y, z coordinates are in MNI space.
**The cluster listed is in the right posterior midbrain; however, an adjacent subthreshold cluster was present in the right substantia nigra.*

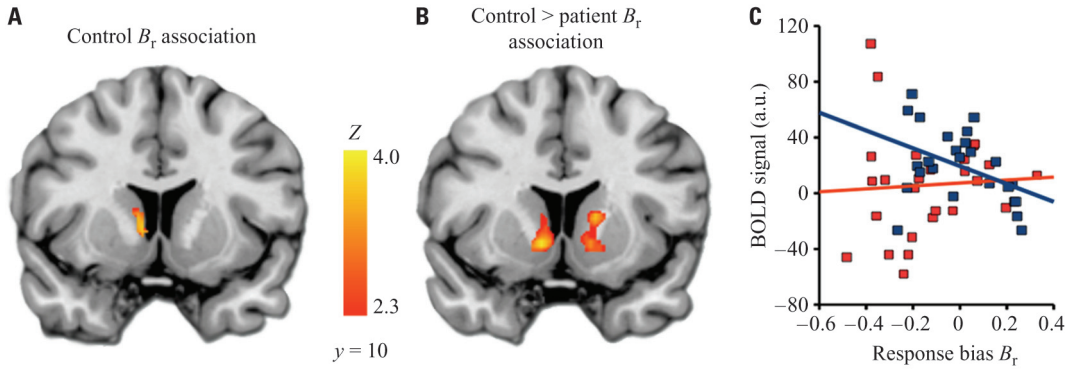

FIGURE 4 | Relationship between ventral striatum activation and response bias. (A) Cluster in VS ROI showing significant correlation between response bias and BOLD activation to HIT > baseline. An analogous within-group analysis in patients revealed no significant results. **(B)** Clusters showing significant differential correlation (controls > patients) to response bias. **(C)** Descriptive scatterplot showing mean BOLD activation in the cluster from **(A)** and response bias for controls (blue) and patients (red). The Pearson's *r* value for the data shown here is −0.42 for controls, and 0.06 for patients; however, correlation values for data extracted from significant clusters may be biased and should not be used for statistical inference, which has already been conducted in identifying the cluster (Vul et al., 2009). Images thresholded at $Z > 2.3$ and cluster corrected for multiple comparisons with spatial extent $p < 0.01$.

revealed only one significant cluster located in the left VS, which was identical to that found in our ROI analysis. Patients did not activate any regions to a greater extent than controls.

BIAS CORRELATION

Within the *a priori* VS ROI among healthy controls, there was a significant negative relationship between B_r and left VS activation to HIT > baseline (**Figure 4**). This indicates that control subjects who were less likely to identify a given stimulus as a target had higher activation of the striatum when they correctly identified a target. Patients did not show a significant correlation with B_r in the striatum. When directly compared, this produced a significant differential association with B_r in bilateral VS between groups.

SPECIFICITY ANALYSIS

The contrast of HIT > MISS in healthy controls produced a pattern of activation similar to the results of the analysis of the HIT > baseline contrast, including activation of bilateral striatum, insula, and dorsal anterior cingulate, with subthreshold anterior midbrain activation. However, patients did not activate these reward-related regions. Direct comparison of controls and patients revealed a significant control > patient response in the right caudate and insula, among other regions (**Table 3**).

POTENTIAL CONFOUNDS

To assess whether the group difference in VS activation was driven by group differences in performance accuracy, we examined the relationship of P_r to VS. P_r did not correlate with target correct responses in VS in either the control or the patient group. Inclusion of P_r as an across-group covariate (thus statistically accounting for group differences in P_r) did not alter the significance of group differences in VS activation or B_r correlation. Thus, VS findings were not confounded by P_r effects. Including or excluding P_r and B_r from the models did not change the above within-group or between-group findings for HIT > baseline or HIT > MISS. Gender, age, and smoking status did not relate significantly to VS activation nor did modeling their effects alter the significance of reported findings.

DISCUSSION

Our results confirm earlier reports that VS and anterior caudate respond preferentially to successful recognition (von Zerssen et al., 2001; Han et al., 2010). We extend this finding beyond prior literature by identifying a relationship of VS activation to response bias, and by demonstrating a deficit in these responses in patients with schizophrenia.

VS RESPONDS TO CORRECT RECOGNITION WITHOUT EXPLICIT FEEDBACK OR REWARD

Extensive literature documents activation of VS to external rewards. However, research examining VS responses reflecting intrinsic motivation is quite sparse. Prior studies have demonstrated that performance feedback activates VS (Rodriguez et al., 2006; Tricomi and Fiez, 2008; Daniel and Pollmann, 2010). Here we tested the hypothesis that striatal reward signals would be present even in the absence of explicit feedback, reflecting monitoring and evaluation of responses by intrinsically motivated subjects. The VS response we observe to correct targets is consistent with this hypothesis as well as the findings of Han et al. (2010). In that study, the anterior caudate response to correct targets occurred in the absence of external rewards or feedback, and was further enhanced when a monetary incentive was provided for correct target recognition. While Han et al. (2010) reported a peak of activation in anterior caudate dorsal to VS, the activated striatal regions overlapped with those observed in this study. Furthermore, Han et al. (2010) found that activation in VS itself correlated most strongly with personality measures of reward sensitivity. While our study is not a direct replication of Han et al. (2010), the differences in methods make the similarities in our findings all the more striking.

Table 3 | HIT > MISS whole-brain significant clusters.

Voxels	Region	Max-*Z*	*x*	*y*	*z*
Controls					
715	dACC	3.39	−6	18	20
634	L frontal pole	3.37	−46	42	−4
604	L lateral occipital cortex	3.61	−30	−92	16
459	R caudate/thalamus	3.89	10	12	−2
449	R insula	4.21	36	20	−12
396	L insula	3.63	−30	24	−6
358	L IFG	3.17	−46	−70	−6
317	L caudate/thalamus	3.55	−12	2	10
308	R MFG	2.94	52	24	20
261	R IFG	3.65	48	8	34
223	R intracalcarine	3.43	10	−86	−4
201	R superior parietal	3.4	34	−54	38
170	L MFG	3.18	−40	32	20
Patients					
598	L supramarginal gyrus	3.29	−52	−34	48
262	L superior parietal	3.54	−30	−52	54
210	L precentral gyrus	3.33	−28	−10	42
180	L IFG	3.61	−54	2	22
Controls > patients					
310	R insula	3.62	34	24	−14
280	R lingual gyrus	3.23	12	−74	−8
272	Cuneus	2.9	−4	−72	20
243	L lingual gyrus	3.26	−12	−60	4
242	R fusiform	3.68	30	−38	−18
219	R lateral occipital cortex	3.18	34	−76	46
181	R caudate/thalamus	3.24	14	14	−2
158	L fusiform	3.11	−24	−88	−4

Data thresholded at $Z > 2.33$ and corrected for multiple comparisons using AlphaSim with a spatial extent $p < 0.01$. Peak-Z x, y, z coordinates are in MNI space.
OFC, orbitofrontal cortex; MFG, middle frontal gyrus; IFG, inferior frontal gyrus; dACC, dorsal anterior cingulate cortex.

VS RESPONSE IS CORRELATED WITH RESPONSE BIAS

By conceptualizing VS responses within the framework of reward prediction errors, we successfully predicted the novel finding that subjects with a more conservative response bias would activate VS more during correct retrieval. Importantly, this result was not driven by discrimination accuracy, which was accounted for in our model. Prediction errors reflect the difference between actual and expected outcome values. Expected outcome value depends on the subjective value of the outcome and its probability of occurring. In contrast, actual outcome value depends on subjective value and the probability that the outcome *has actually been obtained*. In typical reward tasks feedback is given, making the actual outcome certain. However, in a task like the current one with no feedback, the probability that the desired outcome has occurred (here, a HIT) corresponds to subjective confidence in one's response. Therefore, VS prediction error signals should be enhanced by a higher subjective confidence in the correctness of a given response. A conservative response bias could therefore increase prediction errors in two ways. First, subjects with a conservative response bias will have lower expectations that any given stimulus will be a correctly identified target, thereby lowering the expected outcome value. Second, subjects with a conservative bias will therefore tend to report recognition only when they have high confidence they are correct (higher actual outcome value). Thus, subjects with a conservative response bias will have low expectations of correctly identifying a target on any given trial, but higher response confidence will generate a greater outcome value when a target is indeed correctly identified. Together, these two factors could produce greater positive prediction errors. Consistent with this account, conservative bias in the current study strongly correlated both with reduced target hit rate (fewer correct identifications) and with an increased proportion of target responses that were in fact correct. Thus, a subject with a very conservative response bias was unlikely to respond "old" to a previously displayed target (reducing hit rate), but had a high likelihood of being correct when she *did* respond "old."

One reason that such an effect has not been previously described may stem from the difficulty of the task. Greater task difficulty lowers pre-response confidence, and also may increase the subjective value of correct responses, producing greater prediction errors responses in VS. The task used here was quite challenging, as encoding occurred incidentally during brief presentations of abstract visual fractals as oddball distractors, without any expectation of a memory test. Old vs. new recognition tasks used in prior fMRI studies are typically designed to be easier, in order to reduce the number of errors (which are typically unanalyzed), and to ensure all subjects perform at greater than chance levels. We are currently testing the prediction that greater task difficulty enhances VS response to correct responses in a follow-up study.

VS IS PART OF AN INTRINSIC REINFORCEMENT NETWORK

Our ROI analysis focused on the VS based on its central importance in reward and motivation. An exploratory whole-brain analysis revealed a network of other regions previously associated with reinforcement learning that were also strongly activated to successful retrieval, including midbrain, insula/orbital frontal cortex, and dorsal anterior cingulate cortex (Kirsch et al., 2003; Sescousse et al., 2010; D'Cruz et al., 2011). Importantly, activation of these regions was not due to non-specific elements of the task, as these regions were also recruited in the contrast of HIT > MISS. The activation of this network, which is not typically associated with memory retrieval *per se*, further supports our contention that VS responses seen here reflect reward signaling. These findings underline the importance of reward-related circuits in understanding performance and brain responses in cognitive tasks.

PATIENTS WITH SCHIZOPHRENIA HAVE DIMINISHED VS INTRINSIC REINFORCEMENT RESPONSE

The hypothesized deficit in VS activation to correct targets in the schizophrenia group was confirmed. Notably, the result from the VS ROI analysis was also the single statistically significant group difference in the exploratory whole-brain analysis. Multiple fMRI studies have examined recognition memory tasks in schizophrenia patients, identifying abnormalities in various cortical and subcortical regions (Weiss and Heckers, 2001; Achim and Lepage, 2005; Leavitt and Goldberg, 2009; Ragland et al., 2009). To our knowledge prior recognition memory studies have not identified VS deficits. This may be a consequence of using easier tasks (to reduce group performance differences) that do not activate VS robustly even in controls. In addition, in studies where group differences in VS activation are less robust than ours, a VS ROI approach might have been necessary but has generally not been utilized. However, VS hypofunction in schizophrenia is commonly observed in tasks involving reward (Juckel et al., 2006; Wolf et al., 2008; Waltz et al., 2009, 2010). Two studies have reported striatal hypofunction in schizophrenia using different cognitive tasks without an explicit reward component. Koch et al. (2008) reported reduced dorsal striatum activation in schizophrenia during successful working memory retrieval, whereas Vink et al. (2006) reported diminished anterior caudate response in both schizophrenia patients and unaffected family members during a response inhibition task. Although these findings were not framed as deficits in intrinsic reinforcement, both these prior studies and the current results are consistent with such an interpretation. Taken together with the existing literature, the current study provides additional evidence of striatal dysfunction in schizophrenia, demonstrates its occurrence during a recognition memory task, and suggests that such dysfunction may be related to deficits in reward-related processing.

In addition to demonstrating reduced VS activation to correct recognition, schizophrenia patients also failed to demonstrate the increase in VS responses with more conservative bias that was found in controls. This occurred despite a similar average and range of response bias in the patient and control groups. This suggests a failure to generate prediction errors in response to changes in expectation and confidence, and is consistent with substantial evidence of blunted prediction error responses in schizophrenia (Corlett et al., 2007; Murray et al., 2008; Waltz et al., 2009; Romaniuk et al., 2010; Gradin et al., 2011; Morris et al., 2011).

Evidence of blunted VS reward responses in schizophrenia, particularly in those with prominent negative symptoms (Juckel et al., 2006; Wolf et al., 2008; Waltz et al., 2009, 2010), leads us to speculate that the abnormal VS responses seen here reflect reductions in intrinsic motivation and self-generated reward signals. Deficits in intrinsic motivation have been found in schizophrenia and related to clinically relevant outcomes. In particular, Nakagami et al. (2008) demonstrated that intrinsic motivation, neurocognitive performance, and psychosocial functioning are all inter-related in schizophrenia, and that intrinsic motivation mediated the relationship between neurocognition and psychosocial functioning. Similarly, Choi and Medalia (2009) have shown that an intervention aimed at increasing intrinsic motivation in schizophrenia patients improved performance in a difficult cognitive task. Reward and motivation impairments may therefore contribute to cognitive abnormalities in schizophrenia. Our results extend this literature, and suggest VS dysfunction may be a key neural mechanism.

ALTERNATIVE INTERPRETATIONS AND LIMITATIONS

While our findings are consistent with a prediction error framework, certain alternative interpretations and limitations should be acknowledged. First, anterior caudate responses have been observed during successful response inhibition and response switching (Vink et al., 2005; Li et al., 2008; Cameron et al., 2009). These responses have been interpreted as evidence that anterior caudate is important for overcoming prepotent responses. One can conceptualize "old" responses in the setting of a difficult memory task and a conservative response bias as requiring inhibition of the prepotent "new" response. This response inhibition theory would also predict stronger VS responses in those with greater conservative bias. However, it is unlikely that the response inhibition account fully explains VS activation to correct recognition responses, as we have observed stronger VS activation to correct than incorrect responses in the absence of feedback in a facial recognition memory paradigm (Wolf et al., 2011) that utilizes simultaneous target and foil presentations and therefore does not involve response inhibition (unpublished VS ROI analysis).

Second, while the presence of apparent reward prediction signals in the absence of external rewards or explicit feedback is one of the most interesting aspects of the study, this design is also a limitation as it does not directly vary reward outcomes. Future studies can bolster such interpretations by including additional task conditions that directly manipulate rewards, feedback, and task difficulty. In addition, future research should also assess relevant subjective states and traits not measured in the current study, including pre-response confidence, post-response confidence, subjective value of correct and incorrect responses, trait reward sensitivity, and intrinsic motivation.

Third, the number of correct targets available for fMRI analysis was relatively small (∼15 on average in each group), which may have decreased the reliability of within-subject activation estimates. However, this within-subject variance is accounted for in the imaging analysis, and while it may reduce the statistical significance of our results, it is unlikely to explain them. Regardless, future studies would benefit from the increased statistical power afforded by a greater number of trials, as well as a larger number of subjects.

Fourth, group differences in task performance present a potential confound. As in this study, schizophrenia patients' performance in cognitive tasks is typically impaired. If VS prediction error responses are more robust in control subjects when task difficulty increases, there is a tension between achieving adequate patient performance and capturing the phenomenon of interest. However, groups did not differ in accuracy or reaction time for the target recognition condition that was the focus of this study. Furthermore, discrimination accuracy did not correlate with VS responses within either group and inclusion of P_r as a confound covariate across groups did not attenuate the group

difference. Therefore, group differences in recognition performance are an unlikely explanation for observed VS differences.

Finally, our patient population was treated with antipsychotic medications, which are known to affect dopamine signaling in brain regions including VS, and which may affect reward processing, motivation, and cognition in complex ways. The observation of striatal hypofunction in unaffected family members as well as patients (Vink et al., 2006), suggests a vulnerability phenotype rather than an effect of frank illness or medication. Furthermore, given that most schizophrenia patients require long-term treatment with antipsychotics, a potential role of medication effects on VS hypofunction does not negate its importance. Nonetheless, clarifying the role of medications will be important and will require further studies including in drug-naïve populations.

CONCLUSION

Taking into account the above limitations, these findings add significantly to our understanding of normal VS responses during successful retrieval, and dysfunction in these responses in schizophrenia. Examining VS responses during cognitive tasks from the perspective of reward prediction errors can yield new approaches to understanding cognitive–emotional interactions that are critical to healthy functioning as well as psychopathological states. As cognitive deficits and motivational deficits are both disabling and treatment-resistant symptoms of schizophrenia, further investigation of VS dysfunction should prove illuminating and may yield novel approaches to pharmacological and psychological intervention.

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Enhanced training protects memory against amnesia produced by concurrent inactivation of amygdala and striatum, amygdala and substantia nigra, or striatum and substantia nigra

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Memory is markedly impaired when normal activity of any of a number of cerebral structures is disturbed after a learning experience. A growing body of evidence indicates, however, that such interference with neuronal function becomes negligible when the learning experience is significantly enhanced. We now report on the effects of enhanced training on retention after temporary inactivation of cerebral nuclei known to be involved in memory, namely the substantia nigra (SN), striatum (STR), and amygdala (AMY). When training was conducted with a relatively low intensity of footshock (1.0 mA), post-training infusion of lidocaine into the SN, STR, or AMY produced a marked memory deficit. Increasing the aversive stimulation to 2.0 mA protected memory from the amnesic effect of intranigral lidocaine, but there was still a deficit after its infusion into the STR and AMY. Administration of lidocaine into each of these nuclei, in the groups that had been trained with 3.0 mA, was completely ineffective in producing alterations in memory consolidation. Simultaneous infusion of lidocaine into STR + SN, AMY + SN, or AMY + STR was also ineffective in altering memory formation when the highest footshock intensity was used for training. To our knowledge, this is the first demonstration that an enhanced learning experience guards against memory deficits after simultaneous temporary interruption of neural activity of brain nuclei heretofore thought to be necessary for memory formation. These findings support the proposition that brain structures involved in memory processing are functionally connected in series during memory consolidation and that, after an enhanced learning experience, these structures become functionally connected in parallel.

Keywords: memory consolidation, amnesia, TTX, inhibitory avoidance, amygdala, striatum, substantia nigra

INTRODUCTION

Ample evidence indicates that interference with normal activity of any one of a number of cerebral structures brings about deficiencies in memory consolidation and has led to the assumption that a set of cerebral nuclei is essential to establish memory for particular types of tasks. It has been shown, for example, that interference with the activity of the amygdala (AMY) (Liang et al., 1982; Power et al., 2000; Roesler et al., 2000; McGaugh, 2002; Roozendaal et al., 2009), striatum (STR) (Prado-Alcalá et al., 1972, 1973; Sanberg et al., 1978; Prado-Alcalá, 1995; Salado-Castillo et al., 1996; Ambrogi-Lorenzini et al., 1999; Packard and Knowlton, 2002; White and McDonald, 2002; Izquierdo et al., 2006; White, 2009; Miyoshi et al., 2012), and substantia nigra (SN) (Routtenberg and Holzman, 1973; Kim and Routtenberg, 1976; Ambrogi-Lorenzini et al., 1994; Da Cunha et al., 2001, 2003; Díaz del Guante et al., 2004) causes significant deficiencies in retention of inhibitory avoidance (IA). There are data that indicate, however, that treatments which normally induce amnesia become ineffective when infused into these structures in rats that have been subjected to enhanced IA training (Giordano and Prado-Alcalá, 1986; Pérez-Ruíz and Prado-Alcalá, 1989; Parent et al., 1992, 1994, 1995; Parent and McGaugh, 1994; Prado-Alcalá, 1995; Cobos-Zapiaín et al., 1996). This protective effect of enhanced training has also been observed in cats (Prado-Alcalá and Cobos-Zapiaín, 1977, 1979) and rats (Prado-Alcalá et al., 1980) trained to perform operant responses in order to obtain positive reinforcers.

These findings have led to two models that postulate that those cerebral structures involved in memory consolidation in standard conditions of training are functionally connected in series, that is, the neural activity derived from the learning experience must flow through all of them before reaching a hypothetical integrative "center" whose activation is necessary for consolidating memory. This flow is halted when any component of this ensemble of structures is not functional and thus, consolidation is

not achieved. The nature of the integrative center is unknown—it may be one particular cerebral structure, a fixed system of structures, or a number of structures involved in a probabilistic fashion. The second model hypothesizes that, in conditions of learning mediated by enhanced training (relatively high levels of positive or negative reinforcers, a high number of trials or training sessions, or some combination of these factors), those structures that were originally connected in series undergo a functional change whereby they become functionally reconnected in parallel (additional structures may become involved in this process). Consequently, even when one or several components of this circuit are damaged or do not function normally, the neural activity produced by the learning experience will be able to reach the putative integrative center, thus allowing for memory consolidation to occur (Prado-Alcalá, 1995). This model makes an important prediction, namely, that after an enhanced training experience, interference with the activity of one structure (as previously shown), as well as simultaneous interference with several structures will not produce the expected mnemonic deficiencies that are commonly seen in conditions of regular training.

Although there is a fair amount of literature germane to the participation of AMY, STR, and SN in memory processes, there are, to our knowledge, no data that determine the relative importance of these three regions for memory formation or if they participate differentially in these processes. If it turned out that there was a differential effect of typical amnesic treatments on retention, or if enhanced training did not protect against amnesia after simultaneous interference with two structures known to participate in memory, then a major revision of the serial/parallel model described would be in order. Thus, one objective of this work was to test if under identical conditions of training with low, medium, and high aversive stimulation, temporary inactivation of AMY, STR, and SN would produce the same retention deficits of IA. A second goal was to determine if concurrent inactivation of two cerebral areas (AMY + STR, AMY + SN, and STR + SN) would interfere with retention of enhanced training of IA.

MATERIALS AND METHODS

All experimental procedures were approved by the Animal Ethics Committee of the Instituto de Neurobiología, Universidad Nacional Autónoma de México and complied with "Principles of laboratory animal care" of the National Institutes of Health.

ANIMALS

The subjects were naive male Wistar rats (250–350 g) maintained in a room with a 12 h/12 h light-dark cycle (lights on at 7:00 h) and housed individually in acrylic cages with food and tap water *ad libitum*. The temperature of the various rooms in which they were reared and maintained was a constant 21°C. The subjects were randomly assigned to each group; sample size ranged from 10 to 12 rats per group.

SURGICAL PROCEDURE

The rats were adapted to the laboratory vivarium for at least one week before surgery. They were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), given atropine sulphate (1 mg/kg, i.p.), and positioned in a stereotaxic instrument (Stoelting, Co.; IL). Stainless steel guide cannulae (23 gauge) were bilaterally implanted in the STR (A = Bregma, $L = \pm 3.0$, $V = -4.0$), AMY ($A = -2.8$, $L = \pm 4.5$, $V = -8.0$), or SN ($A = -5.3$, $L = \pm 2.0$, $V = -7.5$), or in two of these structures (Paxinos and Watson, 1997). The cannulae were fixed to the skull using two screws and dental acrylic, and a stylet was inserted into each cannula and retained there at all times except during the injections. Immediately after surgery, the animals received an intramuscular injection of penicillin (6000 units). The rats were allowed one week to recover from the surgical procedures before behavioral training, and they were handled on each of the three days preceding training to habituate them to the infusion procedure and maintain patent cannulae.

APPARATUS

Training and testing were carried out in an apparatus specifically designed to study one-trial step-through IA training. The apparatus is an alley with two distinct compartments, separated by a guillotine door. The safe compartment (30 × 30 × 30 cm) had walls and lid of red-colored acrylic with a floor of aluminium bars (6 mm in diameter, 9 mm apart). This compartment was illuminated by a 10-Watt light bulb located in the center of its lid. The other, unlit compartment has side walls and floor made of stainless steel with end walls and lid made of red-colored acrylic. The compartment is 30 cm long and 25 cm deep. The walls and floor are shaped as a trough, 20 cm wide at the top and 8 cm wide at the bottom. In the middle of the floor, a 1.5 cm slot separates the two stainless steel plates that make up the walls and floor. When in this compartment, the rats are in contact with both plates, which can be electrified and, thereby, deliver aversive stimulation consistently to every subject. A square-pulse stimulator (Grass model No. S-48), in series with a constant current unit (Grass model No. CCU-1A), generated the electric shock. The duration of shock and the measurement of latencies to cross from one compartment to the other were accomplished with automated equipment. The conditioning box was located inside a dark, sound-proof room, provided with background masking noise.

TRAINING AND TESTING

On the day of training, each animal was put inside the safe compartment; 10 s later the door between compartments was opened, and the latency to cross to the other compartment was measured (training latency). When the animals crossed to this compartment the door was closed and a footshock was delivered; intensities will be specified below. Five seconds later the door was reopened allowing the animal to escape to the safe compartment, and the stimulator was turned off; this latency was also measured (escape latency). After 30 s in the safe compartment the animal was put back in its home cage. Twenty-four hours later, during the retention test, the same procedure was followed except that the footshock was not delivered; if the animal did not cross to the second compartment within 600 s, a retention latency score of 600 was assigned and the session ended.

MICROINJECTION PROCEDURE

The bilateral infusions of 2% lidocaine or isotonic saline solution were made through 30 gauge injection needles connected to a

Hamilton microsyringe by polyethylene tubing (1 μl into the STR and 0.5 μl into the AMY and SN; at the dose and volumes used here, lidocaine suppresses neuronal activity for less than two hours; Martin, 1991). The injection needles were inserted into the guide cannulae and protruded 1 mm beyond the tip of the cannulae. The infusion rate was 1.0 μl/min and was controlled by an automated microinfusion pump (WPI, model 220i). At the end of the infusion, the injection needles remained inside the guide cannulae for 60 s to insure diffusion away from the injector tip. The injection procedure was carried out within 1 min after training in a different room from that in which training and testing took place.

HISTOLOGY

Upon completion of testing, all rats were anesthetized and perfused, intracardially, with isotonic saline followed by a 10% formalin solution (FS), and their brains were removed and post-fixed with a 10% FS for two days, at which time the solution was replaced with a 10% FS-30% sucrose solution until sectioning. Sections (50 μm thick) were cut on a cryostat and stained with cresyl-violet. Data from animals with cannulae tips outside the target areas were not included in the statistical analyses. **Figure 1** depicts the location of cannulae tips of all successfully implanted rats.

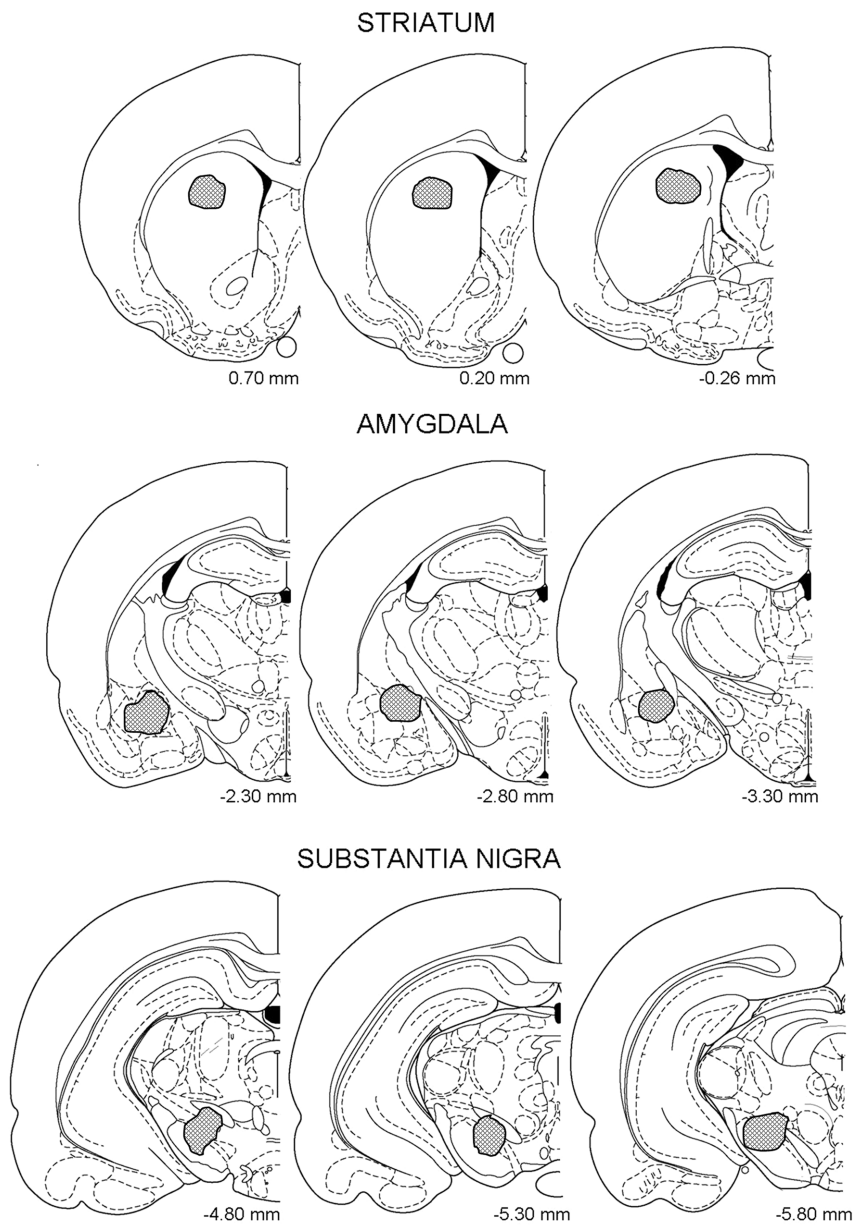

FIGURE 1 | Hatched areas represent location of cannula tips within the striatum, amygdala, and substantia nigra. Redrawn from Paxinos and Watson (1997).

STATISTICS

Because the measurement of retention was truncated at 600 s, non-parametric statistics were used in analyzing the results. Independent Kruskal–Wallis analyses of variance were computed for training, escape, and retention latencies. When appropriate, the Mann–Whitney *U* test was used to make comparisons between any two groups.

EXPERIMENT 1

As stated above, post-training interference with activity of STR, SN, or AMY produces marked retention deficits of IA, but enhanced training protects against these deficits (Thatcher and Kimble, 1966; Giordano and Prado-Alcalá, 1986; Pérez-Ruíz and Prado-Alcalá, 1989; Parent et al., 1992, 1994, 1995; Parent and McGaugh, 1994; Prado-Alcalá, 1995; Cobos-Zapiaín et al., 1996). It is not known, however, whether these structures are equally important for retention of IA. To put it another way, does inactivation of each of these structures produce the same degree of memory impairment? Our results indicated that such is not the case: a greater deficit in retention is produced by temporary inactivation of SN than that produced by inactivation of AMY or STR, although enhanced training overcomes such deficits, as shown below.

The rats of three independent groups that had been implanted in the STR, AMY, or SN, received a 1.0 mA footshock during training; half the rats were microinjected with lidocaine and the other half with isotonic saline.

RESULTS

Because all the groups shared identical conditions during the training (microinjections were delivered after training), data of training and escape latencies of the six groups were analysed with separate Kruskall–Wallis analysis of variance tests, and no significant differences among the groups were found ($H[5] = 3.81$, $p = 0.58$ and $H[5] = 3.645$, $p = 0.60$, respectively). The lack of significant differences in training and escape latencies among the groups was also found in the remaining experiments.

Evaluation of the retention latencies also indicated that there were no significant differences among the saline groups ($H[2] = 0.20$, $p = 0.90$) nor among the lidocaine groups ($H[2] = 0.08$, $p = 0.96$). When pairwise comparisons were made (Mann–Whitney *U* test), however, highly significant differences became evident between the saline- and lidocaine-treated rats of the SN, STR, and AMY groups; **Figure 2** shows these results.

EXPERIMENT 2

Experiment 1 showed that lidocaine infusions produced a marked impairment of memory consolidation, regardless of whether the infusions had been made in the SN, STR, or AMY. The aim of this experiment was, as stated earlier, to find out if increasing the intensity of the training experience, by increasing the magnitude of the aversive stimulation, would produce a differential effect on retention of the task, depending on the cerebral structure whose neural activity had been hindered by the lidocaine. The same experimental design as that of Experiment 1 was followed, except that the intensity of the footshock delivered during training was 2.0 mA.

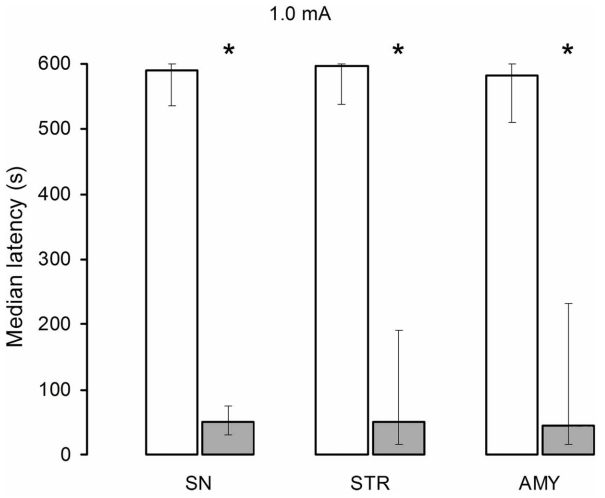

FIGURE 2 | Median retention scores (with interquartile range) of groups of rats trained in one-trial inhibitory avoidance and injected with either isotonic saline solution (open bars) or 2% lidocaine (filled bars). Lidocaine infusion into the substantia nigra (SN), striatum (STR), or amygdala (AMY) produced amnesia in animals trained with 1.0 mA. *Denotes $p < 0.05$ between saline and lidocaine.

RESULTS

While no significant differences in retention scores among the three saline groups were found ($H[2] = 0.05$, $p = 0.97$), the retention scores of the lidocaine groups yielded a significant treatment effect ($H[2] = 9.14$, $p = 0.01$). The *U* tests revealed that there were no significant differences between the effects of saline and lidocaine administration into the SN. In contrast, lidocaine produced a significant retention deficit as compared with the saline treatment in both the STR ($p < 0.001$) and AMY ($p < 0.005$) groups. Finally, pairwise comparisons between the lidocaine groups showed that the AMY and STR groups had significantly lower retention scores than the SN group ($p < 0.01$ and $p < 0.05$, respectively) while the AMY and STR groups did not differ from each other (**Figure 3**).

EXPERIMENT 3

In Experiment 2 it was found that a relatively high footshock during training prevented the amnesic effect of lidocaine infusion into the SN, but that amnesia was still observed when the lidocaine was infused into the STR and AMY. Previous publications had shown that enhanced training of IA protected against the effects of amnesic treatments applied to the SN (Cobos-Zapiaín et al., 1996), STR (Giordano and Prado-Alcalá, 1986; Pérez-Ruíz and Prado-Alcalá, 1989; Prado-Alcalá, 1995), and AMY (Thatcher and Kimble, 1966; Parent et al., 1992, 1994, 1995; Parent and McGaugh, 1994). It was of interest, therefore, to assess the effects of interference with the neural activity of these structures on retention after training with higher footshock intensity, as measured under the same experimental conditions. To this end, rats of three independent groups that had been implanted in the STR, AMY, or SN, received a 3.0 mA footshock during training; half the rats were microinjected with lidocaine and the other half with isotonic saline.

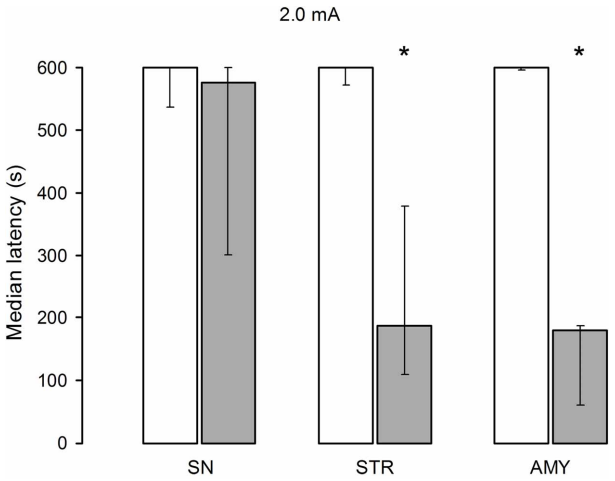

FIGURE 3 | Median retention scores (with interquartile range) of groups of rats trained in one-trial inhibitory avoidance and injected with either isotonic saline solution (open bars) or 2% lidocaine (filled bars). Training with 2.0 mA protected memory in animals infused with lidocaine into the substantia nigra (SN), but not in those infused into the striatum (STR), or amygdala (AMY).
*Denotes $p < 0.05$ between saline and lidocaine.

RESULTS

All groups of rats displayed perfect or near-perfect retention performances, as depicted in **Figure 4**. Independent comparisons of retention latencies among the saline groups and of the lidocaine groups yielded non-significant differences ($H[2] = 0.16$, $p = 0.92$) and ($H[2] = 0.71$, $p = 0.70$, respectively).

EXPERIMENT 4

An important goal of this experimental series was to empirically test the prediction of the proposed "parallel model" (Prado-Alcalá, 1995) that after an enhanced training experience, simultaneous interference with several structures will not produce the expected mnemonic deficiencies that are commonly seen in conditions of regular training. Accordingly, rats were fixed with bilateral cannulae in two structures: STR + SN, AMY + SN, or AMY + STR, and then submitted to the IA task using a 3.0 mA footshock.

RESULTS

Just as in Experiment 3, no significant differences in retention were found among the saline ($H[2] = 3.79$, $p = 0.15$) and lidocaine ($H[2] = 0.34$, $p = 0.84$) groups. The U tests revealed that there were no significant differences between the effects of saline and lidocaine administration in any of the structures (**Figure 5**). It is worth noting that the retention latencies of the AMY + SN and AMY + STR groups treated with saline or lidocaine were somewhat lower than those of the saline groups of Experiments 1, 2, and 3 and the lidocaine groups trained with 3.0 mA (**Figure 5**). These results support the proposition that the STR and AMY are more important for long-term memory formation than the SN.

DISCUSSION

The main finding of this work is that enhanced training protected memory against the amnesic effect of post-training, simultaneous inactivation of any two of three cerebral nuclei known to be involved in memory, namely the AMY, STR, and SN.

Infusion of lidocaine into the SN, STR, or AMY produced a marked memory deficit when training of IA was carried out with the lowest footshock (**Figure 2**). The fact that there were no significant differences in training latencies among the groups indicates that the bilateral implants of cannulae in different cerebral regions did not interfere with the motor and perceptual mechanisms necessary for performance during training. Furthermore, the lack of differences in escape latencies argues against deficiencies in sensitivity to the footshock. These latencies were not significantly

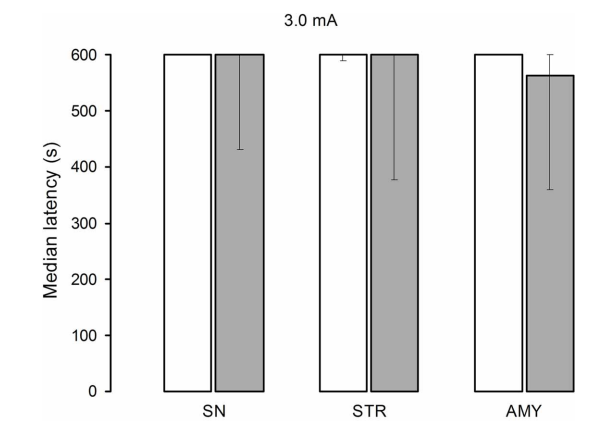

FIGURE 4 | Median retention scores (with interquartile range) of groups of rats trained in one-trial inhibitory avoidance and injected with either isotonic saline solution (open bars) or 2% lidocaine (filled bars) into the nigra (SN), striatum (STR), or amygdala (AMY). Lidocaine was totally ineffective in altering memory when training was conducted with 3.0 mA, regardless of infusion site.

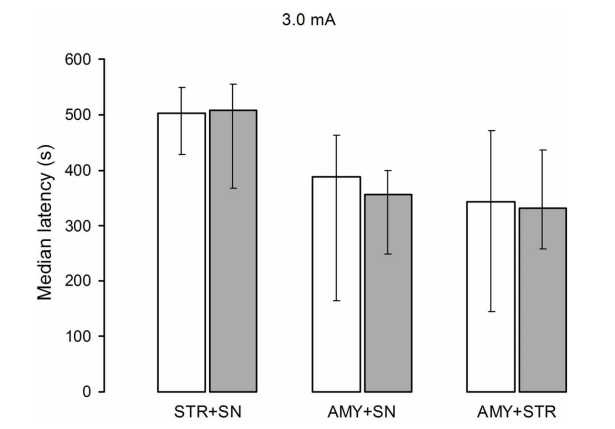

FIGURE 5 | Median retention scores (with interquartile range) of groups of rats trained in one-trial inhibitory avoidance and injected with either isotonic saline solution (open bars) or 2% lidocaine (filled bars). No significant differences among the groups trained with 3.0 mA were found after simultaneous infusion of lidocaine or isotonic saline solution STR + SN, AMY + SN, or AMY + STR.

different from those of a group of unimplanted rats (data not shown). Therefore, the good retention scores of the saline groups and the amnesia shown by the lidocaine groups are attributed to the differential effects of these treatments.

Previous reports had shown, independently, that relatively high intensity of footshock during IA training prevented the effect of a number of amnesic treatments administered to the SN (Cobos-Zapiaín et al., 1996), STR (Giordano and Prado-Alcalá, 1986; Pérez-Ruíz and Prado-Alcalá, 1989; Prado-Alcalá, 1995), and AMY (Thatcher and Kimble, 1966; Parent et al., 1992, 1994, 1995; Parent and McGaugh, 1994). However, there are no direct comparisons among these structures of the effect of interference with their neural activity, using different levels of aversive stimulation in training. In the present work, we made these comparisons under identical experimental conditions. We found that when the aversive stimulation was increased to 2.0 mA, memory was protected when lidocaine was injected into the SN, but there was still a significant deficit after its infusion into the STR or AMY (**Figure 3**). These results indicate that the STR and AMY are more critically involved than the SN in consolidation of the IA task, and that for any given structure the proposed functional transfer from serial to parallel connectivity (Prado-Alcalá, 1995) depends upon the impact of the learning experience.

Administration of lidocaine into the STR, AMY, or SN of the groups trained with 3.0 mA had no effect on long-term memory (**Figure 4**). More importantly, simultaneous infusion of lidocaine into the STR + SN, AMY + SN, or AMY + STR was also ineffective in altering memory formation when a 3.0 mA footshock was administered (**Figure 5**). These results agree well with one of the predictions of the proposed parallel model of enhanced learning (Prado-Alcalá, 1995), namely that after an enhanced training experience, simultaneous interference with several structures will not produce the expected mnemonic deficiencies commonly seen in conditions of regular training.

Not only the overall results of this study support the models described above, but their implications also go to the heart of the current notion about memory formation. The vast majority of experiments dealing with the effects on memory of interference with normal activity of the brain support the century-old theory of memory consolidation (Müller and Pilzecker, 1900). Consistently, it has been found that post-training administration of a variety of treatments shortly after a learning experience produces amnesia. This detrimental effect diminishes as the interval between learning and treatment increases, until the treatments become ineffective (McGaugh, 1966, 2000; McGaugh and Herz, 1972; Weingartner and Parker, 1984; Lechner et al., 1999). However, evidence has accumulated that does not fit the consolidation theory (Prado-Alcalá, 1995). Post-training treatments that produce amnesia of training mediated by aversive stimulation have no effect after enhanced training, independent of the amnesic agents used, and the mode of their administration. It might be argued that these agents do not produce amnesia after enhanced training because memory consolidation has been accelerated and the maximal effect of the pharmacologic agents now occurs after consolidation has taken place. Nonetheless, we have found that *pre-training* infusion of tetrodotoxin into the hippocampus produces amnesia of IA trained with low footshock, but long-term memory was normal when a relatively high footshock was used. These data confirm the protective effect of over-learning against experimentally induced amnesia, and suggest that the establishment of long-term memory was not due to accelerated consolidation (Garín-Aguilar et al., 2003) and that the period of consolidation may be absent in the case of enhanced training. It remains to be determined if this result can be generalized to a condition where infusions of lidocaine are made into the SN, STR, and AMY *before* training.

More experiments are under way to test the validity of these models. If the new data are consistent with the predictions that can be derived from them, then it will be possible to think about the brain as having at least two different ways to store learned information, depending on whether it is dealing with normal or enhanced learning.

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EEG and autonomic responses during performance of matching and non-matching to sample working memory tasks with emotional content

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Working memory (WM) is a memory system responsible for the temporary storage of information and its utilization in problem solving. The central executive is theorized as the controller of storage functions that support WM. Neurophysiological data suggest that electroencephalographic (EEG) theta and alpha oscillations in frontal and midline regions are involved in neural communication between the central executive and storage functions during WM performance. Emotion is known to modulate several memory systems, including WM, through central and peripheral pathways. However, the physiological effect (EEG; autonomic nervous activity) of emotion over WM are not well described. In this study we aimed to identify physiological responses related to emotional WM performance. EEG (21 channels), heart rate (HR), and galvanic skin response (GSR) recordings were obtained from 54 volunteers while performing delayed matching and non-matching to sample tasks (DMTS/DNMTS). Emotional and neutral pictures from the International Affective Picture System and geometric figures were used as stimuli. As expected, WM performance was accompanied by presence of theta (frontal and midline electrodes) and alpha power (parietal electrodes). Beta and gamma oscillations were concentrated in frontopolar and left temporal regions. The DNMTS task was accompanied by higher increases in beta power, HR, and GSR compared to the DMTS task. Correlation analyses showed a positive tendency for gamma in the Fp2 site, ratio of LF/HF and skin conductance in both tasks. The HR results indicate an inverse reaction related to parasympathetic and sympathetic nervous system during the performance of the tasks. Taken together, our results contribute to elucidate the complex interactions between central and autonomic nervous systems in the modulation of emotional WM tasks.

Keywords: emotion, brain dynamics, heart rate, galvanic skin response

INTRODUCTION

Cognition and emotion are two, intrinsically connected, major elements of brain sciences, although often regarded as distinct. Many aspects of real life involve applying some kind of emotional criteria to make a decision or deal with a situation. Earlier studies have shown a high degree of connectivity between brain regions, such as frontal areas and limbic structures, as being central to cognitive–emotional interactions, critical for integration of information from different brain regions (e.g., Young et al., 1994). This relationship between cognition and emotion is performed via many different forms of interaction linked to the functional organization of the brain through many cognitive processes (Phelps, 2006; Pessoa, 2008) and also to body responses through the autonomic nervous system (Scherer, 1984; Lang, 1985; Umeno et al., 2002).

Working memory (WM) is a complex cognitive system designed to retain information until a certain problem has been solved (Baddeley, 1986). The proposal linked to this process is that the resolution of a problem involves the suitability of an overall decision, which requires the evaluation of experienced facts. Moreover, WM alludes to the need to hold a stimulus in a priority state while information is manipulated to reach a certain goal, this being an essential part of the attention process. This focus of attention results in the explicit representation of the information in WM, and the simultaneous inhibition of irrelevant information (Hester and Garavan, 2005; Kim et al., 2005; Korsten et al., 2006; Pratt et al., 2011). The central executive is considered an attention system that controls the explicit manipulation of information during problem solving. It is comprised of the machinery necessary for manipulation of information in collaboration with specific components. One of its servers is the so-called episodic memory buffer, which binds information from multiple systems and recalls memories of personal experiences (Baddeley, 2000).

Electrophysiological measures, such those in electroencephalography (EEG), galvanic skin response (GSR), and heart rate (HR) variability, have been used to evaluate the mental workload and emotion recognition during dual tasks. Some of these studies assess decision-making and perception by means of association between the arousal of emotional content and increases in GSR (Laparra-Hernández et al., 2009). Others use emotional

content to induce changes in autonomic nervous system, especially in HR and in GSR, in an attempt to define standards of autonomic responses for different types of emotion (Lee et al., 2005). Earlier studies indicate that cortical damage, especially to frontal lobes, may selectively attenuate GSR to psychologically relevant stimuli (Zahn et al., 1999). Additionally, cognitive functions such as attention and emotional memory encoding have been related to changes in parasympathetic activity (McCraty and Tomasino, 2006; Critchley, 2009). Some studies found a relationship between frontal and temporal cortice activity and cardiovascular responses associated with increasing emotional intensity, due to high arousal emotional content (Foster and Harrison, 2004).

The WM neural network has been the subject of behavioral, psychophysiological, and functional neuroimaging studies. Neuroimaging and EEG research has revealed the brain connections that support cognition, emotion, and the integration of information. The mainstays of this circuitry are the prefrontal cortex, including the central area, the occipito-parietal, and medial temporal regions (Curtis and D'Esposito, 2003; Osaka et al., 2003; Wager and Smith, 2003; Mizuhara et al., 2004; Erk et al., 2007; Gläscher et al., 2007; Pyka et al., 2009; Michels et al., 2010). Some studies were critical to sustain that the hippocampus modulates the WM system through temporal oscillations (Axmacher et al., 2008; van Vugt et al., 2010). As well as the functional association effects on WM performance, there is a general need to appraise the temporal organization of participant mechanisms using electrophysiological recordings, to demonstrate task-related brain activity (Jensen and Tesche, 2002; Schack and Sauseng, 2005; Kawasaki et al., 2010; Khader et al., 2010; Moran et al., 2010).

Behavioral procedures are important to elicit cognitive processes. The delayed matching to sample (DMTS) and delayed non-matching to sample (DNMTS) paradigms are widely used to assess WM (Tavares and Tomaz, 2002; Winocur and Hasher, 2004; Leiberg et al., 2006) and have been associated with emotional factors (Gasbarri et al., 2008; Satler and Tomaz, 2011). Both tasks involve memory and combining stimuli with a correct response. The DNMTS also requires increased attention to the subject chooses the novel stimulus (Elliot and Dolan, 1999). The difference in performance between stimuli categories, including emotional factors, represents the interaction between emotion and cognition. There is evidence for engagement of frontal and temporal areas of the brain, as well as premotor and occipital cortices, during DMTS and DNMTS performance (e.g., Elliot and Dolan, 1999; Lamar et al., 2004).

Several studies, designed for different purposes, have revealed relationships between cognition/emotion with EEG, HR, and GSR signals. For emotional WM, there are no studies including EEG brain mapping associated with autonomic responses. Therefore, the aim of the present study was to investigate electrophysiological responses during WM performance associated with emotional content processing. Delayed matching and non-matching to sample tasks were used with simultaneous EEG, GSR, and HR recording evaluations.

This approach will serve to elucidate: (1) central and peripheral correlates of WM performance in each of the tasks, (2) central and peripheral correlates of emotional content processing while performing a cognitive tests, and (3) central and peripheral correlates of the interactions between these two factors.

Several hypotheses will be tested: (1) Parasympathetic activity will be higher during DMTS task performance compared to DNMTS; (2) WM performance in both tasks will be facilitated by stimuli with emotionally arousing content; (3) Emotionally arousing content will engage sympathetic activity; and (4) Task and content effects over autonomic activity will interact in conditions of high cognitive demand and emotion arousal (e.g., DNMTS with emotional stimuli).

MATERIALS AND METHODS

PARTICIPANTS

The subjects were 54 young adult (28 females) university students, with an age range from 18 to 28 and mean age of 21.31 years (SD = 2.77). They were healthy, right-handed (assessed by the Edinburgh Inventory; Oldfield, 1971), with no history of neurological or psychiatric episodes. All subjects gave informed consent to a protocol approved by Ethics Committee of the Health Science Faculty, University of Brasilia, Brazil.

DELAYED MATCHING AND NON-MATCHING TO SAMPLE TASK CONDITIONS

This task had two phases in which each trial was intercalated by a delay (1 s). In the first phase, named acquisition, participants were presented with a sample stimulus (size 4 cm × 4 cm) in the center of a computer screen (17″) for 2 s. In the second phase, named testing, participants were randomly presented with two stimuli (4 cm × 4 cm each), one of which was the same as previously presented. In the second phase, the DMTS task required that participants choose the stimulus from the pair that matched the previously viewed target. In contrast, the DNMTS task required that participants choose the novel stimulus from the pair of stimuli after viewing the target (see **Figure 1**). An auditory feedback signal notified the participants if their response was correct (acute, for correct response, or bass, for incorrect or aborted response).

The emotional content of each stimulus was either emotional or non-emotional. Stimuli with emotional content was either Positive or Negative. Non-emotional stimuli was either Neutral or Geometric. Therefore, each stimulus belonged to one of four, mutually exclusive, categories (Positive, Negative, Neutral, or Geometric). The emotional and neutral stimuli were taken from the International Affective Picture System (IAPS; Lang et al., 1999), chosen according to the IAPS scale of valence and arousal. Other simple geometric figures (circles, triangles, etc.) were included to complete the set of stimuli. An equal number of pictures from each category were used. There were a total of 96 stimuli arranged in 48 different pairs, one set for DMTS and another for DNMTS, with the following arrangement: geometric–geometric; geometric–negative; geometric–neutral; geometric–positive; negative–geometric; negative–negative; negative–neutral; negative–positive; neutral–geometric; neutral–negative; neutral–neutral; neutral–positive; positive–geometric; positive–negative; positive–neutral; and positive–positive. These tasks were performed using in house software, written for this purpose in Delphi language.

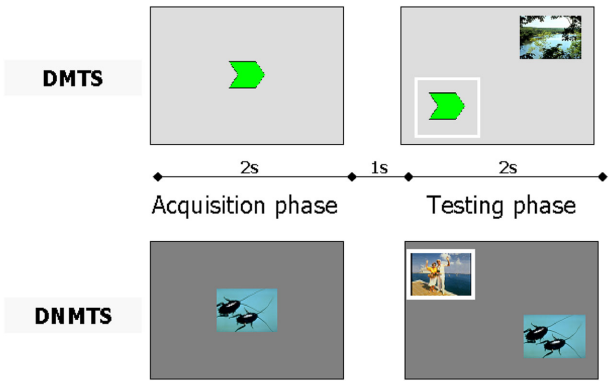

FIGURE 1 | Experimental design using a single trial for delayed matching to sample (DMTS) and another for delayed non-matching to sample (DNMTS). The full procedure included 48 trials for each condition. Each condition was performed with the same parameters. In the acquisition phase, the sample was presented for 2 s followed by presentation of a pair of pictures (sample included) for 2 s in the testing phase, intercalated by a 1-s delay. The participant had to click on the sample picture in acquisition phase, and thereafter, in testing phase, click on the same picture for the DMTS condition, or on the novel picture for the DNMTS condition. The correct response is outlined in white.

DATA ACQUISITION AND PROCESSING

Electroencephalographic data were collected from 21 channels placed on the scalp according to the 10–20 system, plus two reference electrodes on the right and left mastoid, fixed by a conductive paste (Ten20, Weaver and Company, USA). Each electrode site was previously prepared with an abrasive gel (Nuprep, Weaver and Company, USA) to improve conductance. HR was measured through two self-adhesive electrodes: one placed on the neck, over the jugular vein, and another placed on the middle of the left arm. GSR was recorded using two self-adhesive electrodes placed on the left hand, one on the palm and other on the back of the hand. The records were taken simultaneously at a sampling rate of 2000 Hz with an analog bandpass of 0.01–100 Hz using NeuronSpectrum-4/EP system (Neurosoft, Russia). Input impedances were maintained under 5 kΩ during the whole session.

All data were processed using customized Matlab scripts built to digitally separate into non-overlapping epochs time-locked to each task condition. EEG data were analyzed using the open source EEGLAB toolbox, version 9.0.4.5 (Delorme and Makeig, 2004; http://sccn.ucsd.edu/eeglab/). These epochs were submitted to an infomax algorithm to decompose into their independent components (ICA; Bell and Sejnowski, 1995). The components related to eye movement or blinking were removed from the original data, and the record was recalculated using the remaining components. HR and GSR data were filtered and processed for extraction of measures, also with customized Matlab scripts.

Heart rate processing was based on the detection of R peaks to calculate the indices of HR variability – rMSSD, the square root of the sum of the square of differences between adjacent RR intervals; SdNN, SD of RR periods; pNN50, percentage of the total number of all RR intervals of pairs of adjacent RR intervals differing more than 50 ms over the entire recording; LF/HF, ratio of low to high frequency power. The pNN50 helps assess parasympathetic activity: the smaller the percentage, the higher sympathetic activity. LF/HF reflects cardiac autonomic balance, greater values reflect sympathetic dominance, smaller values reflect parasympathetic dominance. GSR data were normalized to enable comparison of the values of each task condition for each subject. The indices calculated were SC, skin conductance mean of GSR values normalized; SCL, skin conductance level; and SCR, skin conductance resistance. These measures reflect sympathetic tone for general response (GSR) or were related to the stimulus presentation.

PROCEDURE

Participation entailed a single session of 2 h duration in the afternoon. By reading and signing the consent form, the participants were invited to make themselves comfortable in a chair in the recording room within a Faraday cage (259 cm × 223 cm × 396 cm), utilized for electromagnetic noise reduction. They were asked to avoid sudden movements or speaking during the task. The test was applied in both conditions, DMTS and DNMTS, in this order for each participant. Electrophysiological recordings were made simultaneously. The software registered correct responses, wrong responses, aborted trials (when participants did not give a response within 3 s) and time taken for responses. The time of execution of the task depended on the time of response of each subject in each of the 48 trials. The room was devoid of luminance and noise during the task performance.

STATISTICAL ANALYSIS

Behavioral data were analyzed using repeated measures ANOVA (Condition × Category, 2 × 4). Statistical analysis on EEG data was performed with EEGLAB Toolbox. The paired t-test was used to compare the condition response for each HR index. A repeated measures ANOVA was also used for GSR data with a paired t-test per condition and stimulus. Degrees of freedom were corrected by Greenhouse–Geisser estimates of sphericity. However, the original degrees of freedom were reported for both ANOVA analyses. Pearson correlation coefficients were calculated among the electrophysiological parameters. Significance was defined as a p value of less than 0.05.

RESULTS

BEHAVIORAL RESULTS

Subjects showed an overall performance of 98.88% correct responses (SD = 1.06) for the DMTS condition and 91.78% (SD = 8.85) for the DNMTS condition (see **Figure 2**). Significant statistical differences were found for performance between conditions (DMTS > DNMTS; $F_{1,53} = 7.697$; $p = 0.008$), between sample stimulus category ($F_{3,159} = 25.632$; $p < 0.001$) and the interaction between condition and stimulus ($F_{3,159} = 27.056$; $p < 0.001$; **Table 1**). Pair-wise comparisons showed statistical differences between Geometric vs. Neutral ($p < 0.001$), Geometric vs. Positive ($p = 0.004$), Geometric vs. Negative ($p < 0.001$), Neutral vs. Positive ($p < 0.001$) sample stimuli. *Post hoc* analyses showed that sample stimulus category effect was absent in DMTS condition ($p = 0.095$). On the order hand, within DNMTS condition the mean of scores of geometric category was significantly

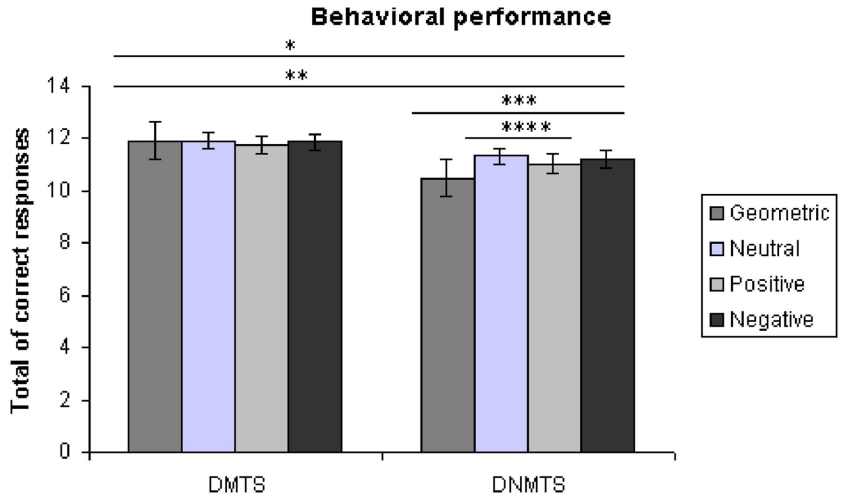

FIGURE 2 | Total of correct answers *DMTS > DNMTS, $p = 0.008$, **geometric < all other categories, $p < 0.005$, ***geometric < all other categories within DNMTS, $p < 0.001$, ****neutral > positive within DNMTS, $p = 0.001$.

Table 1 | Mean ± SEM scores according to task condition and sample stimulus category.

Category	DMTS	DNMTS	Total
Geometric	11.907 ± 0.048	10.481 ± 0.287†	11.194 ± 0.144**
Neutral	11.926 ± 0.045	11.333 ± 0.311††	11.630 ± 0.155
Positive	11.759 ± 0.074	11.019 ± 0.308	11.389 ± 0.154
Negative	11.870 ± 0.046	11.222 ± 0.315	11.546 ± 0.158
Total	11.866 ± 0.036*	11.014 ± 0.301	

**DMTS > DNMTS, $p = 0.008$, **Geometric < all other categories, $p < 0.005$, †geometric < all other categories within DNMTS, $p < 0.001$, ††neutral > positive within DNMTS, $p = 0.001$.*

lower than the other categories ($p < 0.001$), and the mean score for the neutral category was higher than the positive category ($p = 0.001$).]

EEG RESULTS

Electroencephalographic data were filtered and divided into traditional frequency bands: Theta (4–8 Hz), Alpha (8–13 Hz), Beta (13–30 Hz), and Gamma (30–70 Hz). The frequency with the highest activity in each band was selected as the Reference Frequency. **Table 2** shows the mean spectrum power of the frequency with the highest activity in each band. These values cannot be compared between bands, but provide a reference for each one. **Figure 3** shows the topographic maps of activity for each condition.

HR RESULTS

Time domain and frequency domain measures showed significant differences between conditions. DMTS condition induced higher pNN50 ($t = 2.824$; $p = 0.007$), whereas DNMTS induced higher LFHF ($t = -2.673$; $p = 0.010$; **Table 3**). No differences were found for rMSSD or SdNN ($ts < 1.681$; $ps > 0.99$).

Table 2 | Mean spectrum power for each reference frequency.

	Ref. Freq.	Location	DMTS		DNMTS
Theta	5.859 Hz	Fz	7.006	>	6.841
		Cz	6.765	>	6.701
Alpha	9.766 Hz	Fz	3.994	>	3.875
		Cz	4.127	>	4.071
		Pz	3.653	>	3.534
		Fp1	3.356	<	3.901
		Fp2	3.270	<	3.829
Beta	13.67 Hz	Fp1	1.908	<	2.628
		Fp2	1.730	<	2.610
		T3	0.845	>	0.708
Gamma	31.25 Hz	Fp1	−5.034	<	−3.643
		Fp2	−4.803	<	−3.701
		T3	−4.429	>	−4.572

*Theta power was maximal in the midline for both conditions, with maximal expression in the Fz and Cz electrodes. DMTS condition was referenced slightly higher than DNMTS with dampening of this activity in medial regions (F3, F4, C3, C4, P3, P4), as shown in **Figure 3**. Alpha band activity extends toward the parietal central site (Pz) and also frontopolar (Fp1, Fp2) regions, mainly in the DNMTS condition (**Figure 3**) maintaining the expression in the medial regions. Beta power was mostly concentrated in frontopolar regions. This band revealed statistically significant difference between conditions (DNMTS > DMTS; $p < 0.05$) in the Fp2 site. Beta activity was prominent at left temporal electrode (T3) for both groups (**Figure 3**). Gamma band resembled the topography found for Beta activity. Both bands were elevated in the DNMTS condition and highlight an important expression at the left temporal site (T3; **Figure 3**).*

GSR RESULTS

Electrodermal measure did not present any differences, in general, between task condition DMTS: 0.018 ± 0.137, DNMTS: 0.0232 ± 0.137 (mean ± SEM) ($t = -0.031$; $p = 0.975$). Considering the analysis per sample stimulus, significant statistical

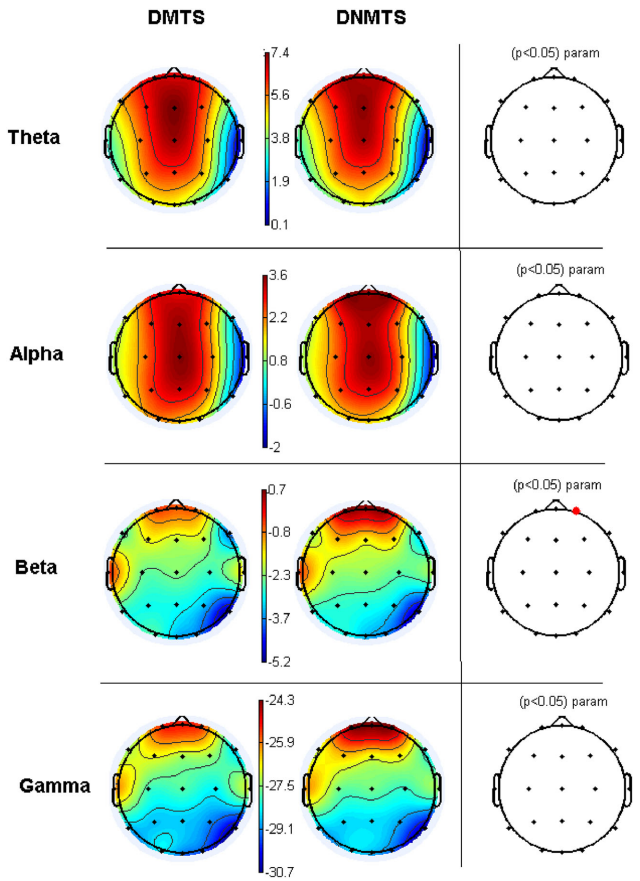

FIGURE 3 | Relative topographic power spectrum distribution for specified bands with each condition task, delayed matching to sample (DMTS) and delayed non-matching to sample (DNMTS), and statistical relevance related to tasks for each location. Theta activity – 4- to 8-Hz; Alpha activity – 8- to 13-Hz; Beta activity – 13- to 30-Hz; Gamma activity – 30- to 70-Hz. Red dot indicates significant difference ($p < 0.05$) in electrode location.

Table 3 | Mean ± SEM of HR values according to task condition.

	DMTS	DNMTS	*p*
rMSSD	0.759 ± 0.015	0.751 ± 0.014	0.099
SdNN	0.048 ± 0.004	0.048 ± 0.005	0.996
pNN50	0.080 ± 0.012*	0.069 ± 0.011	0.007
LFHF	1.628 ± 0.206	1.940 ± 0.214*	0.010

*$p < 0.05$.

differences were found for the SC index in the interaction between condition and sample stimulus ($F_{3,165} = 4.027$; $p = 0.009$). *Post hoc* analyses showed sample stimulus effect in negative category ($p = 0.005$). Statistical differences were marginal for SCL index between conditions (DMTS < DNMTS; $F_{1,55} = 3.754$; $p = 0.058$), and significant in the interaction between condition and sample stimulus ($F_{3,165} = 6.626$; $p < 0.001$). *Post hoc* analyses showed a sample stimulus effect in the geometric ($p = 0.004$) and negative ($p = 0.004$) categories. No significant statistical effect was found between stimuli for SCR index (see **Table 4**).

Table 4 | Mean ± SEM of GSR values according to task condition and sample stimulus.

		DMTS	DNMTS
SC	Geometric	0.1161 ± 0.0468	0.0859 ± 0.0405
	Neutral	0.0981 ± 0.0409	0.1438 ± 0.0390
	Positive	−0.0101 ± 0.1135	0.1892 ± 0.0564
	Negative*	0.2344 ± 0.0600	0.0196 ± 0.0553
SCL	Geometric	0.3457 ± 0.0148	0.3649 ± 0.0141
	Neutral	0.3521 ± 0.0152	0.3512 ± 0.0143
	Positive	0.3534 ± 0.0148	0.3554 ± 0.0139
	Negative	0.3413 ± 0.0150	0.3639 ± 0.0155
SCR	Geometric	0.0004 ± 0.0002	0.0006 ± 0.0003
	Neutral	0.0003 ± 0.0003	0.0009 ± 0.0003
	Positive	0.0031 ± 0.0021	0.0009 ± 0.0003
	Negative	0.0026 ± 0.0015	0.0001 ± 0.0003

*$p < 0.05$.

CORRELATION BETWEEN WM PERFORMANCE AND ELECTROPHYSIOLOGICAL RESPONSES

Pearson bivariate correlations were calculated between all measures outlined above, taking into account an HR, a GSR, and an EEG parameter for all correlations and condition separately. A positive moderate correlation was found between HR (LF/HF) and Fp2 gamma power for DNMTS condition ($r = 0.389$; $p = 0.04$), and between Fp2 gamma power and DNMTS condition score ($r = 0.342$; $p = 0.01$). A weak correlation was seen for DMTS condition between SC and Fp2 gamma power ($r = 0.126$; $p > 0.01$). **Figure 4** illustrates the disposition of these correlations for each task condition. Considering sample stimulus category for performance and autonomic responses, correlations were calculated among scores and GSR's indices. A positive moderate correlation was found between scores and SCL of positive sample stimulus ($r = 0.345$; $p = 0.011$) for DMTS condition. No correlations were found between measures for DNMTS condition.

DISCUSSION

This study investigated EEG power concomitantly with autonomic reactions (GSR and HR) during WM performance associated with emotional content processing.

Behavioral results showed a high performance in both conditions, but statistically significant different scores between them (DMTS > DNMTS). The task comprises the ability to maintain the sample information online and recall this information after a delay according to a specific rule. Participants recalled emotional stimuli, negative and positive, with performance comparable to the neutral and geometric stimuli. The high correct performance found in both tasks limits us to emphasize a possible memory enhancing effect of emotional content, unlike that reported by earlier studies (Kensinger and Corkin, 2003), but consistent with others where negative emotional content were effective over the positive contents (Satler and Tomaz, 2011), mainly in DNMTS condition where more attention is required. However, we found a difference between tasks independent of the stimuli category. This can be explained by the fact that DNMTS requires the choice of the unfamiliar response, inhibiting the instinctively preferred familiar

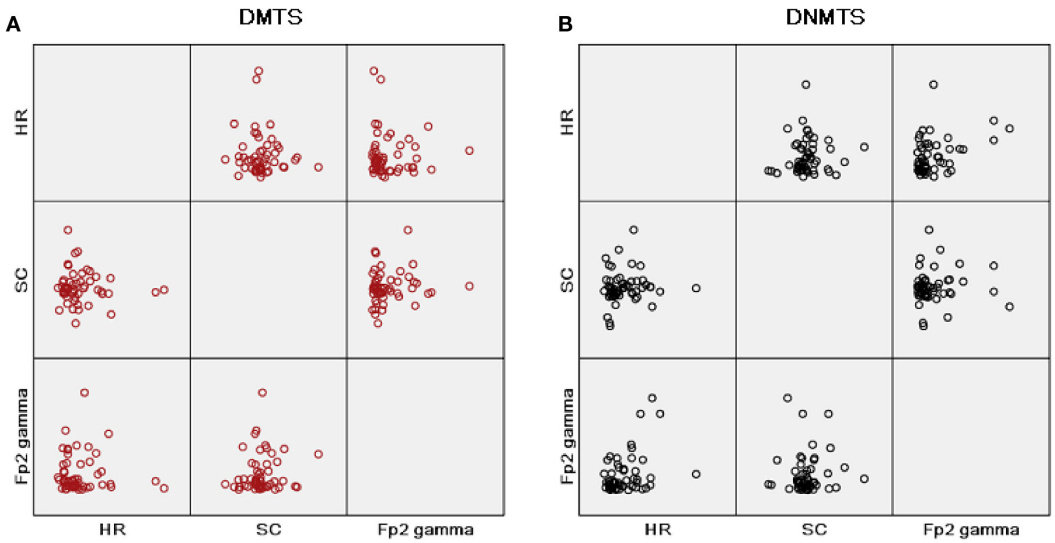

FIGURE 4 | Matrix scatter correlations, heart rate (HR) parameter with ratio of low to high frequency power (LF/HF), skin conductance mean index (SC) and gamma band power at right frontopolar location (Fp2 Gamma), in this order, in rows and columns for each condition task. (A) Condition DMTS; **(B)** condition DNMTS.

response. This aspect makes DNMTS performance lower than DMTS, supposedly due to attention demand (Elliot and Dolan, 1999; Lamar et al., 2004).

Electroencephalographic findings showed diversified rhythms which were present mainly in frontal and midline brain regions. Studies have shown that WM cannot be viewed as a single or dedicated system. A network of brain regions is essential for mediating goal-directed behavior, characteristic of this process (Collette and Van der Linden, 2002; D'Esposito, 2007). Theta activity was mainly observed in the prefrontal cortex and posterior association areas. These areas are strongly associated with central executive actions responsible for manipulation of information and planning (Baddeley, 2002; Jensen et al., 2007). Theta activity may have been induced by the task conditions performed. These results suggest that theta oscillations may play an important role in organizing the information network provided by WM, and this network operates as an integrated unit by means of synchronization in the theta band, consistent with earlier studies (Sarnthein et al., 1998; Raghavachari et al., 2001).

Alpha activity extended from frontal to posterior areas. Earlier findings suggest that alpha, associated with theta, oscillations reflect the transition of information from manipulation to maintenance state of WM tasks (Klimesch, 1999; Schack and Sauseng, 2005; Kawasaki et al., 2010; Moran et al., 2010). Likewise, DMTS tasks reproduces successful memory encoding and loading for processing WM. Increased alpha and theta power are regularly related to WM encoding, considering similar memory encoding processes to increasing WM load. This is consistent with the suggestion that alpha and theta oscillations modulate successful memory encoding (Khader et al., 2010).

The present study shows an increase of beta oscillations in frontal and temporal regions and includes task-related activity on the left hemisphere of the brain. This may be associated with attention demand, as representation of stimuli for DNMTS condition. Frontal beta activation has been thought to be a memory representation of task stimuli (Dörfler et al., 2001; Vernon, 2005; Spitzer and Blankenburg, 2011). This band often appears with theta oscillations, WM load, and encoding processes (Babiloni et al., 2004).

Gamma band activity was distributed over frontal and temporal regions, and higher for DNMTS than DMTS condition. High frequency gamma oscillations have been considered an evidence of involvement in attention process (Mizuhara et al., 2004). Alternatively, gamma activity can reflect memory maintenance according to the short-term memory model, where each item of the memory set is encoded by cortical neurons at the gamma band frequency (Lisman and Idiart, 1995). Previous reports on gamma load-dependence in WM connected with theta oscillations also enhanced the idea that gamma oscillation increases as an expression of maintenance of the to-be-remembered stimuli (Howard et al., 2003; Jensen et al., 2007; Klimesch et al., 2007).

These findings also add some information on WM brain dynamics regarding temporal activity. In previous studies, beta rhythm has been observed at the left temporal and frontal electrodes during WM encoding phase (Onton et al., 2005; Pesonen et al., 2006; Altamura et al., 2010). In a general WM analysis, temporal activity was more prominent in the beta band than the gamma. These results attempt to others that indicate the gamma band leading WM load, with no reference to beta band in any manifestation (Axmacher et al., 2008; van Vugt et al., 2010).

Our results, therefore, show fluctuations in theta, alpha, beta, and gamma bands. Beta band was relevant in right frontopolar site as indicative of consistent task-related changes. Previous studies indicated oscillatory neuronal activity occurring across these frequency bands during WM load (Michels et al., 2010), and these fluctuations were also observed in tasks requiring organization and temporal segmentation of information (Howard et al., 2003).

Some studies have indicated that emotions impact on the memory encoding process (Abe, 2001; Richter-Levin and Akirav, 2003), while underscoring the importance of amygdala–hippocampal connection in emotional memory load (Frank and Tomaz, 2003). Feasible hippocampal projection related to left temporal activity could be favored by subcortical structures responsible for emotion, such as amygdala, so as to better respond to emotional stimuli (McGaugh, 2000). Prior studies with WM tasks showed that bilateral prefrontal cortex activity reflected equally the emotional and WM task components (Gray et al., 2002). Earlier experiments showed a theta band manifestation for emotional content and gamma band for emotion arousal, both in frontal regions (Balconi and Pozzoli, 2009). These studies, along with our results, emphasize a significant electrophysiological interaction between cognition and emotion.

Autonomic responses showed an increased sympathetic activity (high LFHF and low pNN50) for the DNMTS condition. Results in HR index demonstrate a parasympathetic dominance in the DMTS condition, when a lower demand for cognitive functions is expected. Parasympathetic mediation of HRV has been associated with efficient attentional regulation and greater ability to inhibit inappropriate responses. Parasympathetic nervous system control of cardiovascular function, as well as activity of the prefrontal cortex, is associated with these inhibitory processes (Hansen et al., 2003). HR is a physiological variable controlled by sympathetic and parasympathetic activity. However GSR is not influenced by the parasympathetic nervous system, thereby making it a reliable index of sympathetic activity level (Venables, 1991), and is frequently used as an indirect measure of attention, or emotional arousal. The level of skin conductance was consistent with the cognitive effort considering the low performance for geometric sample stimuli and the arousal of negative emotional pictures. Equivalent emotional charge was applied in both conditions, thus no significant differences in GSR were found between conditions, but the expression of the sympathetic nervous system through a cardiovascular response was seen through the increase of GSR in the DNMTS condition.

The role of emotion was analyzed by evaluating human body response by means of HR and GSR measurements. Emotional content somehow modulates WM performance (Kensinger and Corkin, 2003; Levens and Phelps, 2008). Cognitive effort can also exert influence over emotion-related brain activation (Erk et al., 2007). Extrapolating, WM development has some benefit in the comprehension of emotions (Morra et al., 2011). This cross relation suggests that WM is susceptible to emotional content, in the same way that minimizing emotional reactions favors goal maintenance. Thus, the relationship between WM and emotion could be evaluated taking into account the arrangement of electrophysiological responses found while performing a WM task with emotional content. The correlations found between physiological variables were higher in the DNMTS condition, when attention demands are higher.

These results suggest an electrophysiological framework with interactions between brain dynamics and autonomic responses elicited by emotional engagement in a WM task. This adds elements to the relationship between cognitive processes and emotion. Future studies should evaluate the distinction over each emotion valence in task-relevant effects, assessing the specific contribution of positive, negative, and neutral, including abstract, representations.

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Positive and negative emotional arousal increases duration of memory traces: common and independent mechanisms

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We compared the ability of positive and negative emotional arousal to increase the duration of consolidated memory traces. Positive arousal was modulated by manipulating the motivational salience of the testing cage of an object recognition test. Negative emotional arousal was modulated by manipulating shock levels in a step-through inhibitory avoidance (IA). Mice trained in either a high (chocolate-associated) or a low (inedible object-associated) emotionally arousing cage showed discrimination of a novel object 24 h after training, but only mice trained in the more arousing cage showed retention 96 h after training. Mice trained with either low (0.35 mA) or high (0.7 mA) shock intensities showed increased step-through latencies when tested 24 h after training, but only mice trained with the higher shock intensity showed retention of the IA learning 1 week after training. Administration of the phosphodiesterase type IV inhibitor Rolipram immediately after training in the two low arousing conditions increases duration of both responses.

Keywords: long-term memory, persistence, emotional arousal, hedonic valence, object recognition, inhibitory avoidance, cAMP, Rolipram

INTRODUCTION

The establishment of long-term memory (LTM) requires the stabilization of learning-induced synaptic changes. This process is referred to as memory consolidation (McGaugh, 1966; Dudai, 1996, 2002, 2004; Lechner et al., 1999; Dudai and Eisenberg, 2004). However, not all long-term "consolidated" memories last equally. Indeed, everyday experience shows that duration of LTM is highly variable. The behavioral and brain factors underlying this variability are poorly understood.

A large body of evidence indicates that emotional arousal enhances LTM duration by acting on memory consolidation (for review see McGaugh, 2006). However, whether this effect is independent of the hedonic valence (positive or negative) of arousing experience is unknown. Indeed, testing this hypothesis is hampered by the lack of one-trial tasks specifically designed to model the effects of positive arousal on LTM duration. Thus, the first aim of the present study was to develop a behavioral protocol able to induce differentially persistent LTM traces in mice, depending on the magnitude of positive emotional arousal induced by the testing cage during a single learning trial. To this aim, the novel object recognition test (ORT) was used as a one-trial, non-aversive, learning task (Ennaceur and Delacour, 1988; Ennaceur, 2010) and the emotional salience of the testing cage was modulated by repeatedly pairing it with a highly palatable food (chocolate).

Recently, it has been observed that intra-hippocampal infusion of the 8-bromoadenosine-3′, 5′-cyclic monophosphate (8Br-cAMP) analog of cyclic adenosine monophosphate (cAMP) immediately after a weak (low arousing) inhibitory avoidance (IA) training mimics the effect of a strong (high arousing) training by converting a rapidly decaying LTM into a long-lasting one (Rossato et al., 2009). This finding suggests that the enhancement of LTM duration induced by a high emotional arousal may be mediated by cAMP-dependent signaling around the time of memory formation. However, it is unknown whether cAMP-dependent signaling is involved in arousal-induced enhancement of LTM duration regardless of the hedonic valence of the learning experience. Thus, the second aim of the present study was to comparatively evaluate the role of cAMP-dependent signaling in the modulation of LTM duration in both the positive task here reported and in a negative standard one (IA). To this aim, the phosphodiesterase type IV inhibitor Rolipram, known to enhance intracellular cAMP concentration, was administered in a single peripheral injection after low arousing training conditions that induce, in both tasks, only non-persistent LTM traces (i.e., lasting 24 h but not beyond 96 h).

MATERIALS AND METHODS

SUBJECTS

Male CD1 mice (Harlan Laboratories, Udine, Italy) were purchased at 6 weeks of age and housed four to a cage on a 12-h light–dark cycle (lights on at 7:00 a.m.). Food and water were available *ad libitum* except when food deprivation was required by design (see Materials and Methods). Mice were left undisturbed for 2 weeks before behavioral testing. Experiments were carried out in accordance with the Italian national law (DL 116/92) on the use of animals in research.

DRUGS

Rolipram (Sigma-Aldrich, Milano, Italy) was freshly suspended in 5% ethanol and 95% saline solution on every experimental day (Rutten et al., 2006). Rolipram was injected intraperitoneally

(i.p.) in a volume of 10 ml/kg at the following doses: 0, 3, and 10 mg/kg. These doses were chosen because they have been shown to be rapidly effective to elevate cAMP concentration in the mouse brain (Randt et al., 1982). For either IA or object recognition experiments, Rolipram was administered immediately or 120 min post-training.

BEHAVIORAL PROCEDURES

Object recognition

Mice underwent ORT in a custom made apparatus consisting of a square box (60 cm × 60 cm × 30 cm) made of black Plexiglas® subdivided in four equal arenas. The floor of the arenas was made of textured transparent Plexiglas® surmounting a plate of opalescent Plexiglas® thick 1 cm. The apparatus was dimly illuminated by halogen light sources placed below it (Nilsson et al., 2007). Light sources (four) were carefully positioned approximately 50 cm under each corner of the apparatus and directed toward the room floor. This allowed an homogeneous illumination throughout all arenas (∼10 lux, measured with a PCE-EM882 multimeter from http://www.pce-italia.it). The apparatus was placed in a ventilated sound-attenuated cabinet. Objects to discriminate were four types, one type for arena: white wooden cube, gray plastic cylinder, gray plastic sphere, and silver metallic parallelepiped. They were available in triplicate copy and, based on pilot studies, all aroused comparable levels of exploration in CD1 mice (data not shown). For training phase, objects were placed at two opposite corners of the arenas (6.5 cm from the object center to the corner). To prevent mice from displacing objects during testing, they were temporarily fixed to the floor of the arenas with repositionable adhesive pastels (UHU patafix white). On the training trial, mice were allowed to freely explore two identical objects (sample objects: A1 and A2) for 15 min. On the test trial, the third copy of the familiar object (A3) and a novel object (B) were placed in the same location of training and mice were allowed to freely explore them for 10 min. Cohorts of four mice belonging to the same cage and to the same experimental group were tested simultaneously. Each mouse was tested only once. All combinations and locations of objects were counterbalanced to reduce potential biases because of preference for particular locations or objects. Between each session, apparatus and objects were thoroughly cleaned with 70% ethanol to remove urine and fecal boli and to homogenize olfactory trails. Mice behavior was recorded with a camera connected to a Debian GNU/Linux[1] workstation equipped with hardware MPEG encoding capabilities. MPEG videos were blindly analyzed by experienced observers. Object exploration was defined as pointing the nose to the object at a distance of 1 cm and/or touching it with the nose. Turning around, climbing, or sitting on an object was not considered as exploration.

Experiment 1

This experiment was aimed to set-up a model to evaluate the effect of positive emotional arousal on LTM duration in mice and to evaluate the relative role of acute food deprivation and of the caloric and/or fat properties of chocolate in that model.

[1]http://www.debian.org

Effect of positive arousal on long-term memory duration. Sixty-four mice were pre-exposed for 15 h to milk chocolate (Lindt, two pieces for cage, each weighing approximately 2 g) mixed to standard food in the home-cage from 7:00 p.m. of the day before the starting of behavioral testing. Then, mice underwent a delay conditioning phase lasting 4 days. This phase comprised two daily sessions on days 1–3 (10:00 a.m. and 2:00 p.m.) and a single session on day 4 (10:00 a.m.). Sessions were as follows: mice were allowed to freely explore the empty arenas for 10 min then, depending on the experimental group, chocolate pieces (CHOC, $n = 16$) or black Lego® blocks (OBJ, $n = 16$), approximately of the same form and size of chocolate pieces, were dropped in the arenas by the experimenter (one stimulus for arena). Mice were allowed to interact with these stimuli for additional 5 min (total session duration: 15 min). At 7:00 p.m. of day 3 all mice were food-deprived. On day 4 at 2:00 p.m. all mice underwent ORT training. Training session differed from previous ones only for the presence of sample objects in the arenas. Immediately after training, mice were returned to their home cages and they were given *ad libitum* food. Half mice from each group were tested for sample object recognition 24 h after training, the remainder were tested 96 h after training. At 7:00 p.m. of the day before test session all mice were food-deprived.

Control of the role of acute food deprivation. Forty-eight mice were submitted to the same protocol as the CHOC group except for the following differences: a first group ($n = 16$, FF–FF) was never food-deprived; a second group ($n = 16$, FD–FF) was food-deprived 15 h before training but not before test; a third group ($n = 16$, FF–FD) was food-deprived 15 h before test but not before training. CHOC group was renamed FD–FD when compared to FF–FF, FF–FD, and FD–FF groups. Mice from these groups were tested for sample object recognition 96 h after training.

Control of the role of schedule of chocolate exposure. Thirty-two mice were treated identically to the CHOC group but, half (PRE) received chocolate only during conditioning phase, the remainder (POST) received chocolate only during the last 5 min of training. CHOC group was renamed PRE + POST when compared to PRE and POST groups. Mice from these groups were tested for sample object recognition 96 h after training.

Experiment 2

This experiment was aimed to evaluate the ability of Rolipram to enhance LTM duration in the OBJ condition. Thirty-two mice were randomly assigned to this experiment. Mice were treated identically to the OBJ group of Experiment 1. Three groups of mice ($n = 8$) were administered, respectively, vehicle, 3 or 10 mg/kg immediately after training. One group ($n = 8$) were administered 3 mg/kg of Rolipram 120 min after training. Mice were tested for sample object recognition 96 h after training. Lowest effective Rolipram dose has been previously determined in pilot studies (data not shown).

INHIBITORY AVOIDANCE

Mice were trained and tested in a step-through IA task. The apparatus consisted of a trough-shaped alley (91 cm long, 15 cm deep) divided into two compartments by a retractable door: an

illuminated safe compartment (31 cm long) and a dark shock compartment (60 cm long). Mice were not habituated to the dark compartment before the training trial. On the training trial, each mouse was placed in the lit start compartment facing away from the shock compartment. After the mouse stepped with all four paws into the dark compartment, the retractable door was closed and an inescapable foot-shock (0, 0.35, or 0.7 mA, 50 Hz, 2.0 s) was administered. The mouse was removed from the dark compartment 30 s after termination of the foot-shock. The lowest shock intensity was chosen on the basis of previous studies showing that it is sufficient to induce IA retention for at least 24 h in mice (Baarendse et al., 2008). The highest shock intensity was chosen on the basis of a previous study in mice showing that it induces IA retention beyond 24 h (Boccia et al., 2004). On the retention trial, each mouse was placed into the light compartment with the retractable door open and allowed to explore the box freely. The latency to enter the dark compartment with all four paws was recorded with a timer by a nearby experimenter as a measure of retention. Retention test ended either if the mouse stepped into the dark compartment or if it failed to cross within 180 s. In the latter case, mouse was assigned a score of 180 s. Foot-shock was omitted on retention test. Each mouse was tested only once.

Experiment 3

This experiment was aimed to test in mice the effect of magnitude of negative arousal on LTM duration (Bekinschtein et al., 2007). Ninety-six mice were randomly assigned to this experiment. One-third of mice were pseudo-trained by omitting the shock, one-third were trained with the 0.35 mA shock, and the remainder were trained with the 0.7 mA shock. Half of the mice for each shock condition ($n = 16$) were tested for retention 24 h after training, the other half were tested 1 week after training.

Experiment 4

This experiment was aimed to evaluate the ability of Rolipram to enhance LTM duration in the low shock condition (0.35 mA). Sixty mice were randomly assigned to this experiment. Four groups of mice ($n = 12$) were trained with the 0.35 mA shock, then they were administered vehicle, 3 or 10 mg/kg of Rolipram immediately post-training or they were administered 10 mg/kg of Rolipram 120 min post-training. In order to rule out a non-specific drug effect a fifth group ($n = 12$) was pseudo-trained and it was administered 10 mg/kg of Rolipram immediately after being removed from the dark compartment. All mice were tested for retention 1 week post-training. Lowest effective Rolipram dose has been previously determined in pilot studies (data not shown).

DATA ANALYSIS

Statistical analyses were performed on a Debian GNU/Linux workstation using the R free software environment for statistical computing[2] and its graphical interface R-Commander (Fox, 2005). An error probability level of $p < 0.05$ was accepted as statistically significant.

[2]http://www.r-project.org/

Object recognition

Data from Experiment 1 were analyzed using: (1) two-way analysis of variance (ANOVA) for independent factors to compare CHOC and OBJ mice either at 24 or 96 h post-training; (2) two-way ANOVA for independent factors to compare CHOC mice free-fed (FF) or food-deprivated (FD) either at training or at test; (3) one-way ANOVA to compare mice that, except for initial pre-exposure, received chocolate only during conditioning phase (PRE), only after training (POST), both during conditioning and after training (PRE + POST). In case of significant two-way interaction simple effect analysis was performed with the Student's *t*-test for independent samples (Welch variant, two-tailed). In case of significant overall one-way *F* ratio, ANOVA was followed by Tukey's *post hoc* tests.

Data from Experiment 2 were analyzed using: (1) one-way ANOVA to compare mice treated with different Rolipram doses (0, 3, and 10 mg/kg); (2) Student's *t*-test for independent samples (Welch variant, two-tailed) to compare vehicle-treated mice and mice treated with 3 mg/kg Rolipram 120 min post-training). For all experiments, one sample *t*-tests (two-tailed) were used to determine whether the discrimination index (D.I.) of each experimental group was different from 0 (chance level). Mice showing less than 5 s of total object exploration during ORT test were excluded from analysis (Sik et al., 2003).

Inhibitory avoidance

Because a cut-off of 180 s was imposed during test sessions, the step-through latency was expressed as median and inter-quartile range and analyzed with non-parametric tests. Kruskal–Wallis ANOVA was used when comparing more than two groups. Two-sample Wilcoxon test (two-tailed) was used either when comparing two groups or as *post hoc* test following a significant overall Kruskal–Wallis test.

RESULTS

OBJECT RECOGNITION

Experiment 1

Effect of magnitude of positive arousal on long-term memory duration. Two-way ANOVA for total exploration time did not reveal significant effects nor interaction for factors "stimulus paired with test context" and "inter-trial interval". **Figure 1A** shows the D.I. measured 24 and 96 h after training as a function of stimulus paired with test context. Two-way ANOVA for D.I. revealed a significant interaction between stimulus paired with test context and inter-trial interval ($F_{1,60} = 5.06$, $p < 0.05$). For 24 h test, simple effect analysis did not reveal a significant D.I. difference between CHOC and OBJ groups and one sample *t*-tests revealed that D.I. from both groups was significantly above chance level (CHOC: $t_{15} = 3.56$, $p < 0.005$; OBJ: $t_{15} = 5.46$, $p < 0.0001$). For 96 h test, simple effect analysis revealed that OBJ group had a lower D.I. respect to CHOC group ($t_{30} = 3.02$, $p < 0.01$). Moreover, simple effect analysis for each level of stimulus paired with test context revealed that only the D.I. of OBJ group at 96 h test was lower than that of 24 h test ($t_{30} = 2.42$, $p < 0.05$). One sample *t*-tests revealed that only D.I. from CHOC group was significantly above chance level ($t_{15} = 7.24$, $p < 0.0001$). These results indicate that long-term duration of object memory is enhanced when

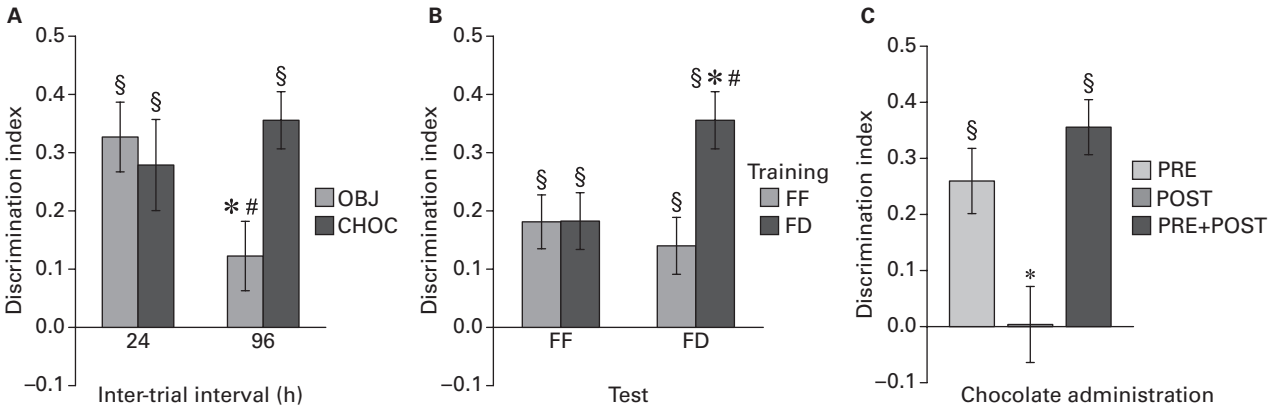

FIGURE 1 | (A) Discrimination index (mean ± SEM) as a function of stimulus paired with test context measured 24 or 96 h post-training (*vs CHOC, $p < 0.01$; #vs 24 h, $p < 0.05$; §vs 0, $p < 0.0001$); **(B)** Discrimination index (mean ± SEM) as a function of feeding state before behavioral testing measured 96 h post-training (*vs FD–FF, $p < 0.05$; #vs FF–FD, $p < 0.005$; §vs 0, $p < 0.0001$); **(C)** Discrimination index (mean ± SEM) as a function of chocolate administration protocol measured 96 h post-training (*vs all, $p < 0.05$; §vs 0, $p < 0.01$).

sampling is performed in an environment previously associated to a rewarding stimulus.

Control of the role of acute food deprivation. Two-way ANOVA did not reveal significant effects nor interaction between factors "feeding at training" and "feeding at test" in total exploration time. **Figure 1B** shows the D.I. measured 96 h after training as a function of feeding state (FF vs FD) before ORT sessions (training vs test). Two-way ANOVA revealed a significant effect of "feeding before training" ($F_{1,60} = 5.06$, $p < 0.05$) and a significant interaction between "feeding before training" and "feeding before test" ($F_{1,60} = 4.93$, $p < 0.05$). Simple effects analysis revealed that: (1) D.I. of mice food-deprived before both training and test (FD–FD) was higher than that of mice food-deprived before training but not before test (FD–FF; $t_{30} = -2.50$, $p < 0.05$); (2) D.I. of mice food-deprived before both training and test (FD–FD) was higher than that of mice free-fed before training but not before test (FF–FD; $t_{30} = -3.11$, $p < 0.005$); (3) D.I. of mice free-fed before both training and test (FF–FF) did not differ from that of mice free-fed before training but not before test (FF–FD); (4) D.I. of mice free-fed before both training and test (FF–FF) did not differ from that of mice free-fed before test but not before training (FD–FF). One sample *t*-tests revealed that D.I. was significantly above chance level for all groups (FD–FD: $t_{15} = 7.25$, $p < 0.0001$; FD–FF: $t_{15} = 3.74$, $p < 0.005$; FF–FD: $t_{15} = 2.86$, $p < 0.05$; FF–FF: $t_{15} = 3.92$, $p < 0.005$). These results indicate that acute food deprivation magnifies the enhancement of LTM duration promoted by sampling objects in the chocolate-associated context.

Control of the role of schedule of chocolate exposure. One-way ANOVA for total exploration did not reveal significant group differences. **Figure 1C** shows the D.I. measured 96 h after training as a function of chocolate administration protocol. One-way ANOVA for D.I. revealed a significant group effect ($F_{2,45} = 7.30$, $p < 0.01$). Tukey's *post hoc* analysis revealed that POST group had a lower D.I. respect to both PRE and PRE + POST groups which did not differ each other. One sample *t*-tests revealed that D.I. was significantly above chance level only for PRE ($t_{15} = 4.46$, $p < 0.001$) and PRE + POST ($t_{15} = 7.30$, $p < 0.0001$). These results indicate that the improvement of object memory observed in mice trained in the chocolate-associated context does not depend on the caloric and/or fat properties of chocolate.

Experiment 2

One-way ANOVA for total exploration did not reveal significant group differences. **Figure 2A** shows the D.I. measured 96 h after training in the object-paired environment as a function of the Rolipram dose injected immediately post-training. One-way ANOVA revealed a significant difference in D.I. between groups

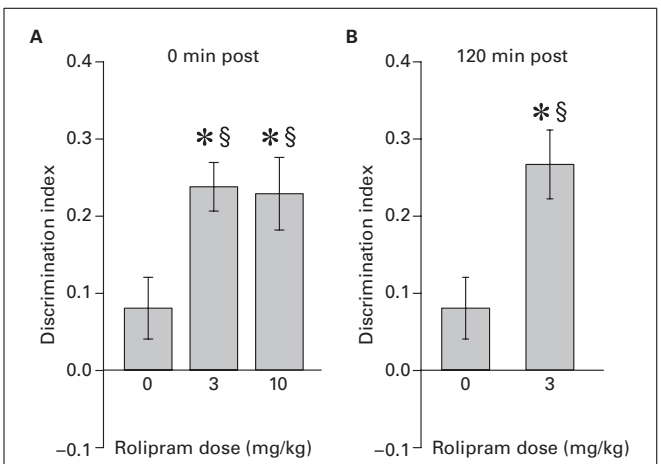

FIGURE 2 | (A) Discrimination index (mean ± SEM) as a function of Rolipram dose (0, 3, and 10 mg/kg) injected immediately after training in the object-paired context measured 96 h post-training (*vs vehicle, $p < 0.05$; §vs 0, $p < 0.01$); **(B)** Discrimination index (mean ± SEM) as a function of Rolipram dose (0 vs 3 mg/kg) injected 120 min after training in the object-paired context measured 96 h post-training (*vs vehicle, $p < 0.01$; §vs 0, $p < 0.001$).

($F_{2,20} = 4.51$, $p < 0.05$). Tukey's *post hoc* revealed that Rolipram enhanced D.I. respect to vehicle at both doses which did not differ one another. One sample *t*-tests revealed that D.I. was significantly above chance level only for Rolipram-treated groups [3 mg/kg ($t_7 = 7.65$, $p < 0.001$); 10 mg/kg ($t_7 = 4.82$, $p < 0.01$)]. **Figure 2B** shows the D.I. measured 96 h after training in object-paired environment in group injected 120 min post-training with 3 mg/kg of Rolipram and in the vehicle group. Two-sample *t*-test revealed that Rolipram-treated mice had higher D.I. respect to vehicle-treated mice ($t_{13} = -3.02$, $p < 0.01$). One sample *t*-test revealed that D.I. was significantly above chance level only for Rolipram-treated mice ($t_7 = 5.91$, $p < 0.001$). These results indicate that Rolipram enhances LTM duration either when injected immediately or 120 min post-training.

INHIBITORY AVOIDANCE

Experiment 3

Figures 3A,B show step-through latency measured, respectively, 24 h and 1 week after training as a function of shock intensity. For 24 h test, Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 23.25$; $p < 0.001$]. *Post hoc* analysis revealed that groups trained with either shock intensities had a higher step-through latency respect to pseudo-trained group but did not differ one another (0.35 vs 0: $W = 26.5$, $p < 0.001$; 0.7 vs 0: $W = 11$, $p < 0.0001$). For 1 week test, Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 13.44$; $p < 0.01$]. *Post hoc* analysis revealed that the group trained with the 0.7 shock had a higher step-through latency respect to both pseudo- and 0.35-trained groups (0.7 vs 0: $W = 41.5$, $p < 0.001$; 0.7 vs 0.35: $W = 197.5$, $p < 0.01$) and that the latter groups did not differ one another. These results indicate that duration of fear LTM depends on the magnitude of negative arousal experienced during training.

Experiment 4

Figure 4A shows step-through latency measured 1 week after training with the 0.35 shock as a function of the Rolipram dose injected immediately post-training. Kruskal–Wallis ANOVA revealed a significant difference between groups [$H_{(2)} = 7.55$; $p < 0.05$]. *Post hoc* analysis revealed that only the group treated with 10 mg/kg of Rolipram had a step-through latency higher than vehicle-treated group ($W = 23.5$; $p < 0.01$). **Figure 4B** shows step-through latency measured 1 week after training with the 0.35 shock in group injected with vehicle or 10 mg/kg Rolipram 120 min post-training. Two-sample Wilcoxon test did not reveal significant difference between groups. **Figure 4C** shows step-through latency measured 1 week after training in groups injected immediately after pseudo-training with vehicle or 10 mg/kg Rolipram. Two-sample Wilcoxon test did not reveal significant difference between groups. Taken together, these results indicate that Rolipram enhances fear LTM only at the highest dose and only when injected immediately after training. Moreover, Rolipram has no effect on LTM duration in absence of shock.

DISCUSSION

Two are the main findings of the present study. The first is that long-term object memory is enhanced when sampling is performed under positive emotional arousal. Indeed, in the present experiments we demonstrated that mice remember a sampled object for 24 h but this memory can last 96 h if object sampling is performed in an environment previously associated with a rewarding stimulus. The second finding is that pharmacological stimulation of cAMP cascade enhances the duration of low emotional memories regardless of hedonic valence. Thus, we found that mice treated with Rolipram post-trial showed discrimination of an object sampled under low emotional arousal 96 h earlier and show IA of a context associated 1 week before with a low intensity shock.

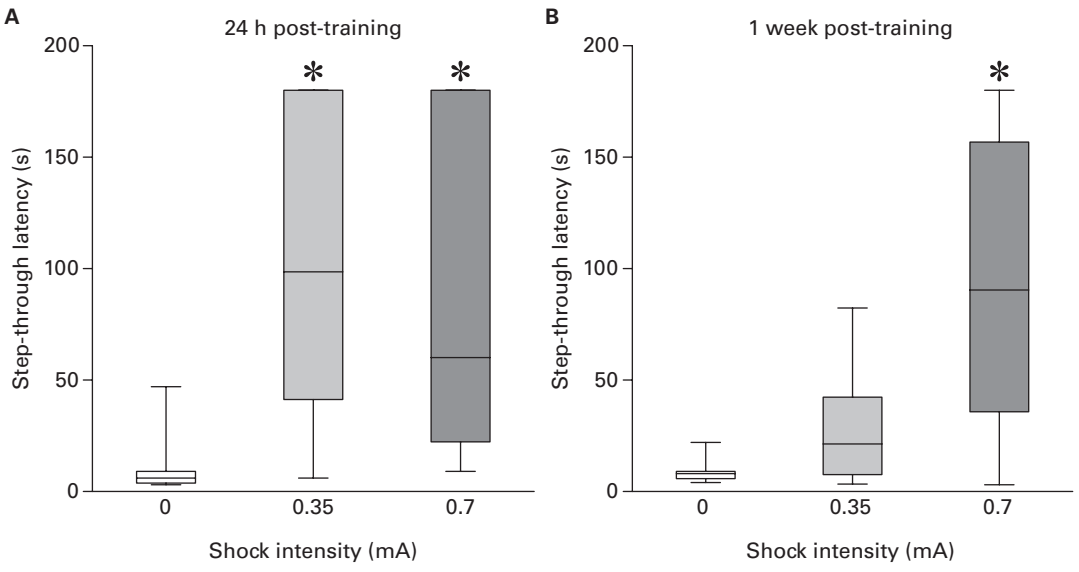

FIGURE 3 | (A) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 24 h post-training (*vs 0, $p < 0.001$); **(B)** Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 1 week post-training (*vs 0, $p < 0.001$).

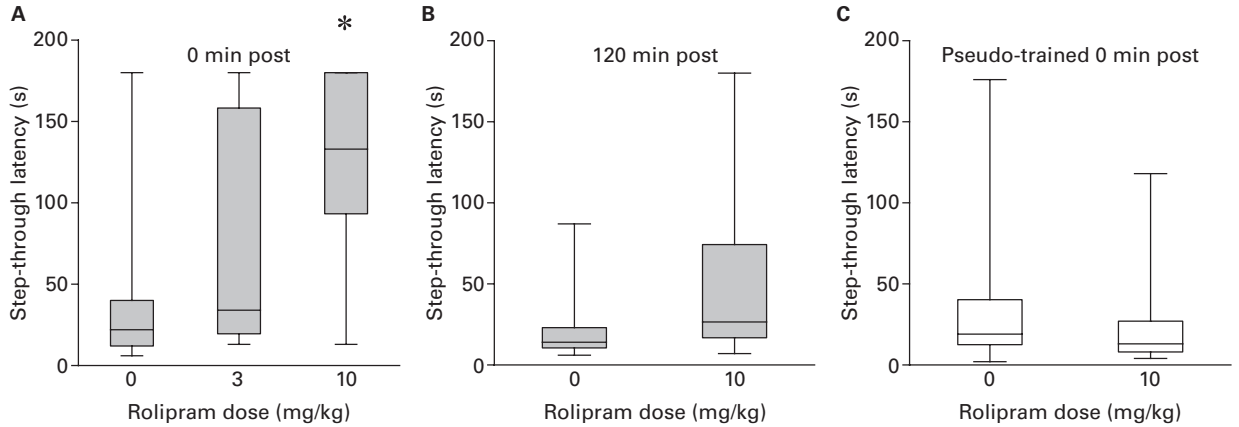

FIGURE 4 | (A) Step-through latency (median and inter-quartile range) as a function of foot-shock intensity (0.35 vs 0.7 mA) measured 1 week post-training (* vs all, $p < 0.01$); **(B)** Step-through latency (median and inter-quartile range) as a function of Rolipram dose (0, 3, and 10 mg/kg) injected immediately after training with the 0.35 mA foot-shock measured 1 week post-training (*vs all, $p < 0.05$); **(C)** Step-through latency (median and inter-quartile range) as a function of Rolipram dose (0 vs 10 mg/kg) injected 120 min after training with the 0.35 mA foot-shock measured 1 week post-training.

POSITIVE EMOTIONAL MODULATION OF LONG-TERM MEMORY

The present study used a modified version of the widely used ORT that allowed modulation of the memory trace acquired during sampling by positive emotional arousal. To this aim we associated the experimental context with chocolate and then increased the motivational salience of the unconditioned stimulus by submitting the mice to a period of food deprivation immediately before ORT training and testing phases. According to several studies on reward anticipation, animals respond with a positive emotional arousal when they are returned into contexts that predicts the availability of highly palatable, caloric, and fat foods (Alcaro et al., 2007; Boissy et al., 2007; Alcaro and Panksepp, 2011). Mice trained and tested in a context associated with an inedible plastic stimulus were used as controls.

Both experimental and control mice discriminated the novel object when tested 24 h after training demonstrating intact memory of the previously sampled object. However, 96 h after training only mice trained and tested within the chocolate-associated context were still able to discriminate the novel object. These data support the view that experiences associated with a state of positive emotional arousal are consolidated in more lasting memories as reported for those associated with negative ones.

Control experiments reported by the present paper indicate that the LTM of the sampled object is modulated by the chocolate-associated context rather than by the chocolate feeding immediately after sampling. Indeed, mice that did receive chocolate on the object sampling session without previous chocolate-context pairing did not show discrimination of a novel object 96 h later, whereas mice that did not receive chocolate but were trained in a context previously associated with chocolate did. These findings rule out the influence of sugar or fat (Campolongo et al., 2009; Smith et al., 2011) on memory consolidation in our experimental conditions. Moreover, in our standard experimental condition chocolate was made available at the end of the sampling session to prevent negative emotional arousal associated with frustration (for review see Flaherty, 1996) as well as contingency between sample object and reward (Hughes, 2007).

Moreover, the data suggest that the chocolate-associated context was capable of promoting a lasting memory of the sampled object also in free-feeding mice; however, food-deprived mice showed a D.I. significantly higher than free-fed mice. This result supports the hypothesis that imposing 15 h of food deprivation before objects sampling increased the positive emotional arousal promoted by chocolate-associated context in line with the hypothesis that a physiological depletion enhances the incentive value of the unconditioned and conditioned goal stimuli (Berridge, 2004). Mice food-deprived before training but not before test showed lower discrimination than mice trained and tested in food deprivation strongly supporting a state-dependent learning. Finally, mice food-deprived only on test day (96 h) did not show any improvement of object discrimination in comparison with free-fed mice. The latter observation indicates a selective effect of the motivational state on memory formation and/or consolidation rather than on retrieval. Taken together, these findings support the view that acute food deprivation increased emotional arousal promoted by the context associated with chocolate, It is tempting to speculate that the condition of feeding deprivation invested the experience of re-entering into the reward-predicting context of a flashbulb-like quality (Brown and Kulik, 1977).

NEGATIVE EMOTIONAL MODULATION OF LONG-TERM MEMORY

Results from IA experiment confirm and extend to the mouse those of Bekinschtein et al. (2007, 2008a,b) in rats. Indeed, we found that training with both foot-shock intensities promoted LTM (i.e., IA at 24 h post-training) but only training with the stronger one induced a persistent LTM (1 week). The facilitating effect of negative arousal on LTM duration is in line with the above described effect of positive arousal, strongly suggesting that emotional arousal enhances LTM duration regardless of hedonic valence.

PHARMACOLOGICAL STIMULATION OF CAMP CASCADE ENHANCES PERSISTENCE OF LOW EMOTIONAL MEMORIES

Pharmacological experiments evaluated the effects of single post-trial administration of Rolipram on retention of low emotional memories. We used two different tests: ORT and step-through IA. For both tests we considered protocols capable of promoting LTM traces of moderate persistence. In the case of ORT, we used mice trained and tested in the context associated with the plastic stimulus because these animals show retention 24 h but not 96 h after training. In the case of IA, we performed a preliminary experiment using 0.35 and 0.70 mA and chose the lower shock intensity that was effective in increasing step-through latencies 24 h but not 1 week post-trial.

The effects of Rolipram were tested at the time-points at which no retention was observable (96 h for ORT and 1 week for IA) in vehicle-treated mice. In both cases we found retention in Rolipram-treated mice. Rolipram is a phosphodiesterase type IV inhibitor that, at the doses used in the present experiments, promotes a rapid rise of cAMP concentration in the mouse brain (Randt et al., 1982). The treatment, in both cases, was performed immediately after a single training session, when the memory traces are under consolidation and susceptible of manipulation (McGaugh, 1966). Therefore, the present results suggest that an increase of brain cAMP concentrations during consolidation mimics the effects of high emotional arousal on persistence of long-term memories.

Nonetheless, major differences were found in the effects of Rolipram on ORT and IA retention. Thus, only the high dose of the phosphodiesterase inhibitor was effecting in enhancing step-through latencies 1 week after IA training, whereas the lower dose was already capable to enhance D.I. to the level of the high dose in mice tested 96 h after ORT training. The most economic explanation of this difference could be that step-through latencies and D.I. were tested at different time points after training. Indeed, if the pharmacological manipulation is increasing duration of the memory traces, a lower dose could be sufficient to produce a 96-h duration whereas only larger doses would induce 1 week-lasting memories. Another difference between the effects of Rolipram on retention in the two conditions was that the drug improved object memory when administered 120 min post-trial, whereas it improved IA retention only when administered immediately post-trial. It should be noted that Rolipram improves object memory in rats if injected 3 h, but not immediately, after sampling (Rutten et al., 2007). However, the dose administered in the present study is much more higher than the one used by Rutten et al. (2007). Thus, the observed efficacy of immediate post-sampling administration could depend on the half-life of Rolipram extending up to 120 min. On the other hand, such hypothesis fails to explain why an even higher dose (10 mg/kg) enhances IA retention if administered immediately, but not 120 min, after training. This may suggest that newly formed non-aversive memories remain susceptible to cAMP stimulation for a longer time respect to aversive ones.

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Neural mechanisms underlying the induction and relief of perceptual curiosity

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Curiosity is one of the most basic biological drives in both animals and humans, and has been identified as a key motive for learning and discovery. Despite the importance of curiosity and related behaviors, the topic has been largely neglected in human neuroscience; hence little is known about the neurobiological mechanisms underlying curiosity. We used functional magnetic resonance imaging (fMRI) to investigate what happens in our brain during the induction and subsequent relief of perceptual curiosity. Our core findings were that (1) the induction of perceptual curiosity, through the presentation of ambiguous visual input, activated the anterior insula and anterior cingulate cortex (ACC), brain regions sensitive to conflict and arousal; (2) the relief of perceptual curiosity, through visual disambiguation, activated regions of the striatum that have been related to reward processing; and (3) the relief of perceptual curiosity was associated with hippocampal activation and enhanced incidental memory. These findings provide the first demonstration of the neural basis of human perceptual curiosity. Our results provide neurobiological support for a classic psychological theory of curiosity, which holds that curiosity is an aversive condition of increased arousal whose termination is rewarding and facilitates memory.

Keywords: curiosity, fMRI, arousal, memory, reward processing

INTRODUCTION

Curiosity is a basic biological drive in both animals and humans, and has been identified as a key motive for learning and discovery. In the 1950s and 1960s, curiosity and related behaviors were topics of intense investigation among experimental psychologists, resulting in an extensive theoretical framework for understanding curiosity and related behaviors (e.g., Berlyne, 1954, 1960, 1966; Loewenstein, 1994). However, despite the importance of curiosity in many aspects of behavior, little is known about the neurobiological mechanisms underlying curiosity. In the present study, we used functional magnetic resonance imaging (fMRI) to test specific predictions of a classic psychological theory of curiosity, developed by Berlyne (1954). According to this theory, curiosity evoked by ambiguous, complex, or conflicting stimuli is an aversive condition associated with increased levels of arousal. The theory further holds that termination of this condition, through access to relevant information, is rewarding and promotes learning.

We focused on perceptual curiosity, the most basic type of curiosity that is found in animals as well as humans. One way to induce perceptual curiosity is to present subjects with blurred pictures. An early study using this method showed that blurred pictures evoked longer EEG desynchronization (alpha-wave blocking) than clear pictures, but only when the identity of the blurred pictures was unknown, which provides preliminary evidence that perceptual curiosity causes an increase in arousal (Berlyne and Borsa, 1968). Another experiment showed that participants actively preferred to view the clear version of a preceding blurred picture over viewing an unrelated clear picture (Nicki, 1970). Importantly, the preference for uncertainty reduction disappeared when participants knew the identity of the blurred picture. These findings are consistent with the idea that the reduction of perceptual curiosity is rewarding.

We used a modified version of the blurred pictures paradigm to investigate the neural underpinnings of both the induction and the subsequent relief of human perceptual curiosity. More specifically, we examined whether we could find support at the neural level for the main assumptions of Berlyne's theory. First, the assumption that curiosity is an aversive condition of increased arousal predicts that the induction of curiosity will produce activation in brain areas sensitive to autonomic arousal, conflict and other aversive states. The two brain regions that are typically activated by a broad range of aversive conditions (including pain, uncertainty, errors and disgust) are the anterior cingulate cortex (ACC; Ridderinkhof et al., 2004; Shackman et al., 2011) and the anterior insular cortex (AIC; Peyron et al., 2000; Singer et al., 2009); hence we predicted that perceptual curiosity would activate these regions. Second, the assumption that the reduction of curiosity is rewarding predicts that this will produce activation in brain regions involved in reward processing, such as the striatum.

Third, the assumption that the reduction of curiosity promotes learning and memory predicts that uncertainty-reducing stimuli will be associated with enhanced memory performance and increased hippocampal activation.

MATERIALS AND METHODS

PARTICIPANTS

Nineteen healthy volunteers participated (14 women and five men; aged 19–29 years; mean age = 22.8 years; SD = 2.4), in return for €25. All participants gave written consent before participation, and the study was approved by the medical ethics committee of the Leiden University Medical Center. All participants had normal or corrected-to-normal vision and reported to be right-handed.

TASK DESIGN

Participants were scanned while they viewed sequences of two pictures of common objects, in a passive-viewing task. To manipulate the induction and reduction of perceptual uncertainty, we used the following four combinations of clear and blurred pictures (**Figure 1**):

1. A blurred picture followed by its corresponding clear picture ($B–C_{corresponding}$)
2. A blurred picture followed by an unrelated clear picture ($B–C_{unrelated}$)
3. A clear picture followed by its corresponding blurred picture (C–B)
4. A clear picture followed by an identical picture (C–C).

Entropy, an information-theoretic measure of uncertainty, increases with the number of possible outcomes and with the nearness in likelihood of the different possible outcomes (Shannon, 1948). Berlyne (1965, p. 246) proposed that someone's subjective uncertainty about a specific stimulus or event (e.g., the identity of an object or the solution to a problem) depends in a similar way on the number of alternative hypotheses, and the relative confidence placed in each hypothesis. A previous behavioral study using the blurred pictures paradigm has shown that subjective uncertainty (derived from the number of guesses regarding the picture's identity and the relative confidence placed in each guess) was maximal for pictures with an intermediate degree of blur (Nicki, 1970). That study also showed that participants' preference to see the corresponding clear version of a blurred picture was larger for pictures with an intermediate degree of blur than for pictures with either a very low or a very high degree of blur. Based on these findings, we only used blurred pictures with an intermediate degree of blur (i.e., with maximal subjective uncertainty).

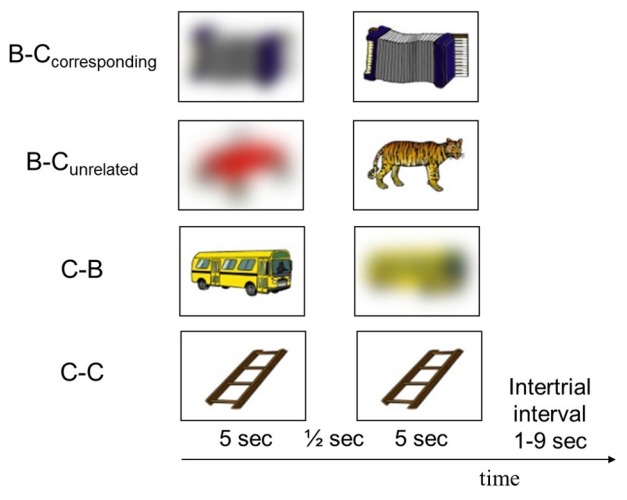

FIGURE 1 | Examples of pictures presented in each of the four conditions. The experiment consisted of 35 trials from each condition, presented in pseudorandom order.

All pictures were selected from Rossion and Pourtois' colored picture databank (Rossion and Pourtois, 2004). This databank is a set of 260 colored line drawings of objects, provided with norms for name agreement, image agreement, familiarity, and complexity ratings. We selected 140 pictures with perfect name agreement from this databank. The pictures had a resolution of 71 dpi, and were centered on a white rectangle of 197 × 281 pixels. We created a blurred version of each picture by means of Gaussian smoothing with a radius of 20–22 pixels (Adobe Photoshop 5.0; all pictures can be found at www.sandernieuwenhuis.nl/SOM). By reducing the picture's high-frequency components, Gaussian smoothing acts as a low-pass filter. Results from a behavioral pilot experiment with 49 participants indicated that the objects displayed in the blurred pictures could not be identified by the majority of the participants.

On each trial, a sequence of two pictures was presented. The pictures were projected onto a screen and viewed through a mirror attached to the head coil of the scanner. Each picture was presented for 5 s in the middle of the screen on a white background, and was surrounded by a black frame (visual angle = 18.5 × 13.8°). The two pictures in a trial were separated by a 500 ms interval during which only the frame was presented. The intertrial interval varied between 1 and 9 s (uniform distribution). The experiment consisted of 35 trials from each of four conditions illustrated in **Figure 1**, presented in pseudorandom order. For the blurred pictures in the $B–C_{unrelated}$ condition we used blurred versions of 35 additional pictures from Rossion and Pourtois' databank (i.e., pictures of which the clear version was not used). The 140 clear pictures were presented in the same order for all participants. To exclude the possibility that differences between the conditions were caused by picture-specific effects, we divided the 140 clear pictures into four subsets of 35 pictures with comparable familiarity, complexity and imagery ratings (all $ps > 0.86$) and alternated the coupling of the four picture subsets to the four conditions across participants according to a balanced Latin-square design.

The experiment was divided into five runs of 28 trials between which we stopped the scanner to verify that the participant was still attending to the pictures. Each run contained seven trials from each condition and lasted approximately 8 min. Participants were not aware of the aim of the study; we told participants that the experiment was designed to investigate the brain activation associated with the perceptual processing of clear and blurred pictures, and informed them of the four possible ways in which clear and blurred pictures could be combined.

After completing the experiment, participants were given an unexpected free-recall test outside the scanner; they were asked to type in the names of as many objects as they could recall from the pictures they had seen in the scanner. Subsequently, participants were asked to indicate, on a five-point scale (1 = not at all; 5 = very much), the degree to which they had (1) been curious about the blurred pictures; (2) tried to guess the identity of the objects depicted on the blurred pictures; (3) been disappointed when a blurred picture was not followed by the corresponding clear version; (4) recognized the objects depicted on the blurred pictures; and (5) tried to remember the pictures. Finally, participants completed the perceptual curiosity scale (Collins et al., 2004).

IMAGE ACQUISITION

Scanning was performed with a standard whole-head coil on a 3-T Philips Achieva MRI system (Best, The Netherlands). In each of the five functional runs, 210 T2*-weighted whole-brain EPIs were acquired (TR = 2.2 s; TE = 30 ms, flip angle = 80, 38 axial slices, 2.75 × 2.75 × 2.75 mm + 10% interslice gap). In addition, a high-resolution EPI scan and a T1-weighted anatomical scan were obtained for registration purposes (EPI scan: TR = 2.2 ms; TE = 30 ms, flip angle = 80°, 84 axial slices, 1.96 × 1.96 × 2 mm; 3D T1-weighted scan: TR = 9.7 ms; TE = 4.6 ms, flip angle = 8°, 140 axial slices, 0.88 × 0.88 × 1.2 mm).

IMAGE ANALYSIS

MRI data analysis was carried out using FMRI Expert Analysis Tool (FEAT) version 5.98, which is part of FMRIB's Software Library (FSL; Smith et al., 2004). Image pre-processing consisted of motion correction (Jenkinson et al., 2002), non-brain removal (Smith, 2002), spatial smoothing using an 8 mm full-width at half-maximum (FWHM) Gaussian kernel, grand-mean intensity normalization of the entire 4D data set by a single multiplicative factor, and high-pass temporal filtering to remove low-frequency artifacts (Gaussian-weighted least-squares straight line fitting, with sigma = 100 s). Functional scans were registered to high-resolution EPI images, which were registered to T1 images, which were registered to standard MNI space (Jenkinson and Smith, 2001; Jenkinson et al., 2002).

The fMRI time series were analyzed using an event-related approach in the context of a general linear model with local autocorrelation correction (Woolrich et al., 2001). We constructed six explanatory variables of interest: two for the first picture in a trial (Blurred or Clear), and four for the second picture in a trial (Clear-corresponding, Clear-unrelated, Clear-double, or Blurred). Each explanatory variable was time-locked to the picture onset and had a duration of 5 s (i.e., the entire duration of the picture presentation). The hemodynamic response to each event was estimated by convolving each explanatory variable with a canonical hemodynamic response function and its temporal derivative. The model was high-pass-filtered (Gaussian-weighted least-squares straight line fitting, sigma = 100 s).

For each run, in each participant, we assessed several contrasts (see Results section). The contrasts were combined across the five runs on a subject-by-subject basis using fixed-effects analyses (Beckmann et al., 2003; Woolrich et al., 2004). These second-level contrast images were submitted to third-level mixed-effects group analyses (Beckmann et al., 2003; Woolrich et al., 2004).

Region-of-interest analyses

We conducted region-of-interest (ROI) analyses to test the predicted AIC and ACC activation in response to the induction of perceptual uncertainty, and the predicted striatal and hippocampal activation in response to the relief of perceptual uncertainty. We used anatomical ROIs of the bilateral insular cortex, ACC (comprised of the anterior cingulate and paracingulate gyrus; Fornito et al., 2006), striatum (comprised of the caudate, putamen and nucleus accumbens), and hippocampus, as specified by the Harvard–Oxford subcortical structural atlas, and implemented in FSLView version 3.1.2. Only the voxels that were part of these areas with a probability of at least 50% were included in the ROIs. The statistical parametric images were thresholded using clusters determined by $Z > 2.3$ and a cluster-corrected significance threshold of $p < 0.05$ (Worsley, 2001). To further examine the hippocampal activation, we extracted the average time course of the hemodynamic response function in response to the second picture in each of the four conditions using PEATE (perl event-related average time course extraction), a companion tool to FSL (http://www.jonaskaplan.com/peate/peate-tk.html). Time courses were extracted from the hippocampal activation clusters of the curiosity-relief contrast (i.e., the regions with stronger activation in response to the second picture in the $B–C_{corresponding}$ condition than in response to the second picture in the $B–C_{unrelated}$ condition).

To examine whether individual differences in trait-perceptual curiosity and free-recall performance were predictive of individual differences in brain activation, we extracted each participant's peak *z* value from the activation clusters of interest. We correlated these peak *z* values with participants' scores on the perceptual curiosity questionnaire and with their free-recall performance. In addition, we computed the across-subject correlations between the peak *z* values of the different activation clusters of interest.

Disappointment median-split analysis

To examine whether participants' rated disappointment when the identity of a blurred object was not revealed predicted the strength of their ACC/AIC activation in response to perceptual uncertainty, we divided all participants into two groups based on their disappointment ratings: nine participants reported a strong disappointment (ratings of 4 or 5 on a five-point scale) and the other ten participants reported less disappointment (ratings of 2 or 3). We used a *t*-test to determine whether the high-disappointment group showed stronger ACC/AIC activation in response to perceptual uncertainty than the low-disappointment group.

Whole-brain analyses

In addition to the ROI analyses, we conducted exploratory whole-brain analyses to examine the activation of brain areas outside our ROIs in response to induction and/or relief of perceptual uncertainty. In these analyses, statistical parametric images for each contrast were thresholded at $p < 0.001$, with a minimum cluster size of 26 MNI voxels (208 mm^3).

Table 1 | Participants' ratings of the degree to which they had been curious about the blurred pictures, recognized the blurred pictures, and had tried to remember the pictures (means ± standard deviations).

I was curious about the blurred pictures	4.11 ± 0.88
I tried to guess the identity of the objects depicted in the blurred pictures	4.53 ± 0.70
I was disappointed when a blurred picture was not followed by its clear version	3.16 ± 1.02
I recognized the objects depicted in the blurred pictures	2.79 ± 0.92
I tried to remember the pictures	1.74 ± 0.73

All ratings were on a scale from 1 (not at all) to 5 (very much).

RESULTS

We scanned 19 healthy participants while they viewed sequences of two pictures, in a passive-viewing task (**Figure 1**). Our task design resulted in the induction of perceptual uncertainty by the first picture on half of the trials (the B–$C_{corresponding}$ and B–$C_{unrelated}$ conditions), which was resolved by the second picture on half of these trials (the B–$C_{corresponding}$ condition). Participants' ratings after the scanning session indicated that they had indeed been curious about the blurred pictures (**Table 1**).

FREE-RECALL PERFORMANCE

The number of pictures that participants recalled in an unexpected free-recall test after the scan session was significantly affected by the condition in which the pictures had been presented [$F(3,54) = 11.5$, $p < 0.001$]. Participants recalled more pictures from the B–$C_{corresponding}$ condition (mean = 10.4, SD = 4.8) than pictures from the B–$C_{unrelated}$ (mean = 6.1, SD = 3.7),C–C (mean = 7.3, SD = 3.8) and C–B (mean = 8.0, SD = 4.4) conditions (all ps < 0.03). Thus, incidental memory for uncertainty-reducing stimuli was enhanced. In addition, participants recalled fewer pictures from the B–$C_{unrelated}$ condition than from the C–C and C–B conditions (ps < 0.04). The number of pictures recalled from the C–C and C–B conditions did not differ ($p = 0.28$).

BRAIN ACTIVATION ASSOCIATED WITH PERCEPTUAL UNCERTAINTY

To examine the brain activation associated with perceptual uncertainty, we focused on the neural response to the first picture in each trial, and identified brain regions where activation was larger when the picture was blurred compared to clear. Our ROI analyses of the ACC and insular cortex revealed significant activation in the ACC (one cluster extending into both hemispheres) and the right AIC (**Figure 2**, upper panel; **Table 2**, upper part). Functional-connectivity studies have suggested that the AIC and AAC are part of a putative "salience network" (Seeley et al., 2007), which has been associated with autonomic arousal (Critchley, 2005) and various aversive emotional experiences (e.g., Craig et al., 1996; Ploghaus et al., 1999; Eisenberger et al., 2003).

The whole-brain analysis for this contrast also revealed activation in the bilateral AIC and ACC, as well as activation in regions of the inferior frontal gyrus, frontal pole, lingual gyrus, occipital pole and posterior cingulate gyrus (**Table 3**).

A whole-brain analysis (cluster-corrected, $p < 0.05$) of the opposite contrast, which identified brain regions that were more

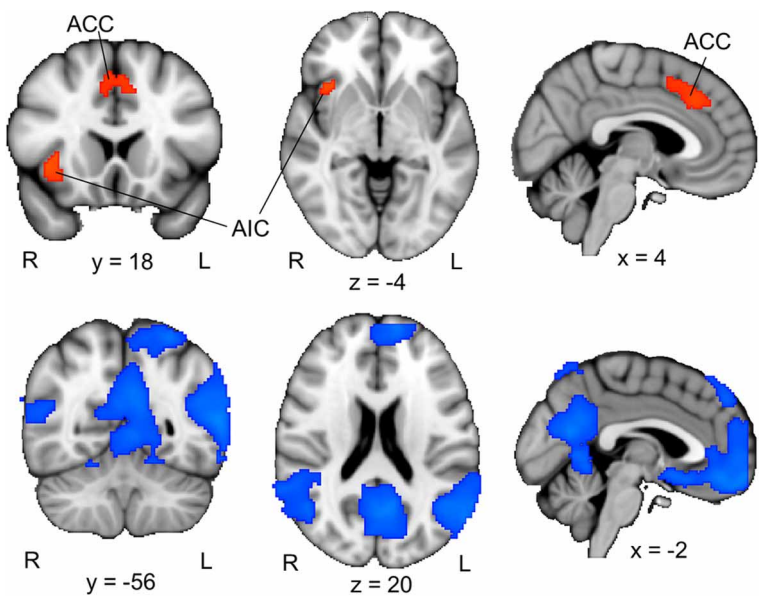

FIGURE 2 | ACC/AIC activation associated with perceptual uncertainty. Upper panel: The colored regions were more active when the first picture in a trial was blurred (i.e., the first pictures in the B–$C_{corresponding}$ and B–$C_{unrelated}$ conditions) than when it was clear (i.e., the first pictures in the C–C and C–B conditions). *R* = right; *L* = left; ACC = anterior cingulate cortex; AIC = anterior insular cortex. The displayed activations are the cluster-corrected *Z* statistic maps ($p < 0.05$) from the ACC/AIC ROI analyses. Lower panel: The colored regions were deactivated when the first picture in a trial was blurred compared to when it was clear. The displayed activations are whole-brain cluster-corrected *Z* statistic maps ($p < 0.05$). All activations are overlaid onto the standard MNI brain.

Table 2 | Activation clusters from the ROI analyses.

Region	Left/Right	Cluster size (mm^3)	Z_{MAX}	MNI peak coordinates (mm)		
				x	*y*	*z*
PERCEPTUAL UNCERTAINTY: FIRST PICTURE BLURRED > FIRST PICTURE CLEAR						
Anterior insular cortex	R	800	4.27	34	22	−4
Anterior cingulate cortex	R/L	3152	3.95	8	28	38
RELIEF OF PERCEPTUAL UNCERTAINTY: SECOND PICTURE IN B–$C_{CORRESPONDING}$ CONDITION > SECOND PICTURE IN B–$C_{UNRELATED}$ CONDITION						
Striatum	L	2672	3.26	−12	6	10
Hippocampus	R	664	2.93	28	−12	−22
Hippocampus	L	1008	3.08	−26	−16	−14

Table 3 | All brain regions that were activated in response to perceptual uncertainty (upper part) and in response to the relief of uncertainty (lower part).

Region	Left/Right	Cluster size (mm^3)	Z_{MAX}	MNI peak coordinates (mm)		
				x	*y*	*z*
PERCEPTUAL UNCERTAINTY: FIRST PICTURE BLURRED > FIRST PICTURE CLEAR						
Anterior insular cortex	R	3192	4.32	36	24	−4
Anterior insular cortex	L	1152	4.11	−28	22	−4
Anterior cingulate cortex	R	1464	4.13	10	24	48
Anterior cingulate cortex	L	488	3.45	−6	12	44
Inferior frontal gyrus	R	3240	4.00	50	16	26
Frontal pole	R	424	3.51	32	48	8
Lingual gyrus	R/L	5520	4.09	8	−80	−8
Occipital pole	L	912	4.21	−12	−94	10
Posterior cingulate gyrus	R/L	616	3.91	2	−30	24
RELIEF OF PERCEPTUAL UNCERTAINTY: SECOND PICTURE IN B–$C_{CORRESPONDING}$ CONDITION > SECOND PICTURE IN B–$C_{UNRELATED}$ CONDITION						
Caudate (dorsal striatum)	L	224	3.26	−12	6	10
Putamen (dorsal striatum)	R	600	3.55	30	−20	10
Orbitofrontal cortex (extending into ventral putamen and insular cortex)	L	624	3.54	−28	6	−12
Lateral occipital cortex	R/L	1808	3.46	40	−74	0
Posterior insula	R	456	3.57	42	−4	6

Data are thresholded at $p < 0.001$ (uncorrected) with a minimum cluster size of 26 contiguous MNI voxels.

activated by clear pictures than by blurred pictures, revealed activation in a set of brain regions that have been associated with the "default-mode network" (**Figure 2**, lower panel). The default-mode network, which includes regions of the precuneus, posterior lateral parieto-occipital cortex and medial prefrontal cortex, is typically stronger activated during rest than during cognitive effort (e.g., Raichle et al., 2001). The relative deactivation of this network in response to blurred compared to clear pictures suggests that participants actively processed the blurred pictures. Consistent with this interpretation, participants indicated that they had been curious about the blurred pictures, had tried to guess the identities of the objects depicted in them, and had been rather disappointed when a blurred picture was not followed by its corresponding clear version (**Table 1**).

Several findings suggest that the AIC/ACC activation reflected a neural substrate of a negative arousal state associated with perceptual curiosity. First, the activated regions of the AIC and ACC closely overlap with areas that are typically activated in response to errors, negative feedback and other aversive events (Ullsperger et al., 2010). Second, the strength of participants' AIC activation was positively correlated with their trait curiosity as indexed by the perceptual curiosity questionnaire ($r = 0.54$, $p = 0.02$; **Figure 3**). Third, the participants who reported more disappointment when the identity of a blurred picture was not

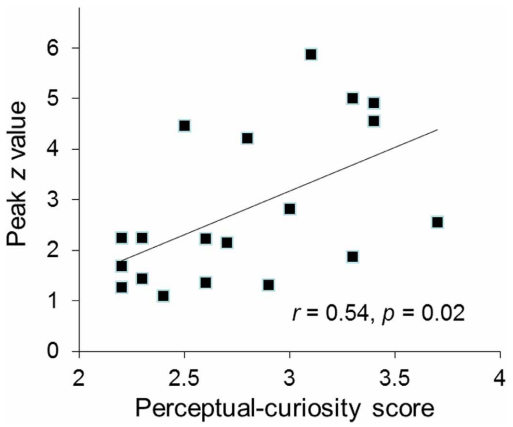

FIGURE 3 | The individual participants' peak activation (*z* value) for the perceptual-uncertainty contrast in the right AIC plotted against their perceptual curiosity score.

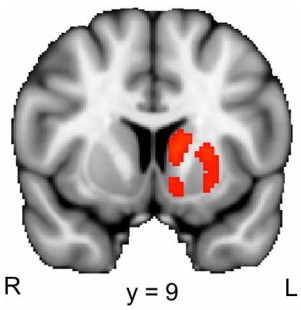

FIGURE 4 | Striatal activation associated with the reduction of perceptual uncertainty. The colored regions were more active when the second picture in a trial reduced the perceptual uncertainty induced by the preceding picture (i.e., the second picture in the B–$C_{corresponding}$ condition) than when the second picture did not reduce the perceptual uncertainty induced by the preceding picture (i.e., the second picture in the B–$C_{unrelated}$ condition). *R,* right; *L,* left. The displayed activations are the cluster-corrected *Z* statistic maps ($p < 0.05$) from the striatal ROI analysis, which were overlaid onto the standard MNI brain.

revealed showed stronger ACC activation than the participants who reported less disappointment [$t(17) = 2.4$, $p = 0.04$; see Section "Disappointment median-split analysis"].

Interestingly, the strength of participants' ACC activation associated with perceptual uncertainty was predictive of the number of pictures they later recalled from the B–$C_{corresponding}$ condition ($r = 0.51$, $p = 0.03$; there was also a trend for a positive correlation between the numbers of pictures recalled from the B–$C_{corresponding}$ condition and the AIC activation, $p = 0.075$), but not of the number of pictures they recalled from the other conditions (all $ps > 0.28$). This suggests that the uncertainty-related activation of the ACC contributed to the enhanced memory for stimuli that reduced this uncertainty.

BRAIN ACTIVATION ASSOCIATED WITH THE RELIEF OF PERCEPTUAL UNCERTAINTY

To examine the brain activation associated with the relief of perceptual uncertainty, we created a contrast that identified brain regions where activation was larger in response to the second picture in the B–$C_{corresponding}$ condition than in response to the second picture in the B–$C_{unrelated}$ condition. Our ROI analysis of the striatum revealed a significant cluster of activation that encompassed regions of the left caudate, putamen and nucleus accumbens (**Figure 4**; **Table 2**, lower part). These activated areas have been associated with reward processing, the coding of "reward-prediction errors" (i.e., the difference between actual and expected reward) and reinforcement learning (O'Doherty, 2004; Daw and Doya, 2006; Haruno and Kawato, 2006). Since the uncertainty induced by a blurred picture was relieved by the following picture on only half of the trials, the reduction of perceptual uncertainty by the second picture possibly caused a (partial) reward-prediction error. Accordingly, the striatal activation could reflect the reward value and/or the reward-prediction error associated with the relief of perceptual uncertainty.

Confirming predictions, the ROI analysis of the hippocampus revealed that regions of the bilateral hippocampus showed stronger activation in response to the second picture in the B–$C_{corresponding}$ than in the B–$C_{unrelated}$ condition (**Figure 5**). A contrast that identified brain regions where activation was larger in response to the second picture in the B–$C_{corresponding}$ condition than in the C–C condition also revealed significant activation in the bilateral hippocampus (512 and 88 mm^3 in the left and right hippocampus, respectively). The event-related time courses of the BOLD signal in response to the second picture in each of the four conditions illustrate the specific increase in hippocampal activation for the B–$C_{corresponding}$ condition (**Figure 5**). The increased hippocampal activation in response to uncertainty-reducing stimuli likely underlied the enhanced later recall of these stimuli. Interestingly, the strength of participants' hippocampal activation in response to the reduction of uncertainty was positively correlated with the strength of their AIC activation in response to the induction of uncertainty ($r = 0.57$, $p = 0.01$ and $r = 0.53$, $p = 0.02$ for the left and the right hippocampus, respectively).

The whole-brain analysis for this contrast also revealed striatal activation, as well as activation of the lateral occipital cortex, orbitofrontal cortex and posterior insula (**Table 3**).

DISCUSSION

The present results provide the first demonstration of the neurobiological basis of human perceptual curiosity. By elucidating the neural underpinnings of the induction and relief of perceptual curiosity, our study extends existing behavioral accounts of curiosity. In particular, our results are consistent with Berlyne's classic psychological theory of curiosity (Berlyne, 1954, 1960, 1966). First, our finding that perceptual uncertainty activated brain regions sensitive to arousal and conflict supports the assumption that curiosity evoked by ambiguous stimuli is an aversive condition, and induces an increase in arousal. Second, our finding that the reduction of perceptual uncertainty activated striatal regions involved in reward processing supports the assumption that the termination of this condition, through access to relevant information, is rewarding. Third, our findings

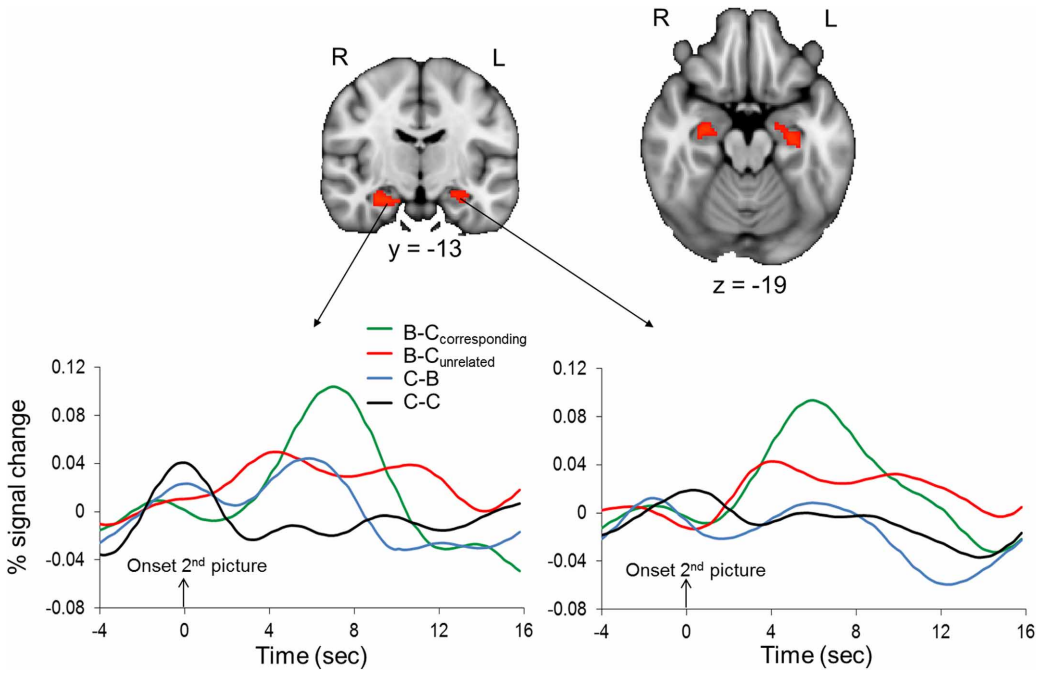

FIGURE 5 | Hippocampal activation associated with the reduction of perceptual uncertainty. Upper panel: the colored regions were more active when the second picture in a trial reduced the perceptual uncertainty induced by the preceding picture (i.e., the second picture in the B–$C_{corresponding}$ condition) than when the second picture did not reduce the perceptual uncertainty induced by the preceding picture (i.e., the second picture in the B–$C_{unrelated}$ condition). The displayed activations are the cluster-corrected *Z* statistic maps from the hippocampal ROI analysis ($p < 0.05$) overlaid onto the standard MNI brain. Lower panel: Time course of hemodynamic activity in response to the second picture in each of the four conditions. Time courses were extracted from the hippocampal activation clusters shown in the upper panel.

that the reduction of perceptual uncertainty was associated with increased hippocampal activation and enhanced incidental memory support the assumption that uncertainty reduction facilitates memory and learning.

Our findings are also consistent with Loewenstein's information-gap account of curiosity which proposes that curiosity is a negative feeling of deprivation that is caused by an inconsistency, or gap, between one's actual and aspired level of knowledge (Loewenstein, 1994; see Litman et al., 2005, for empirical tests and a more detailed investigation of this theory). Since people differ in their aspired level of knowledge, the same actual level of knowledge will evoke curiosity in some people but not in others. In line with this idea, we found that inter-individual variation in trait perceptual curiosity correlated with the strength of AIC activation in response to perceptual uncertainty, suggesting that people with a higher level of aspired perceptual knowledge experience stronger negative feelings when confronted with ambiguous perceptual input.

The relief of perceptual curiosity was associated with activation in regions of the striatum (left caudate, putamen and nucleus accumbens) involved in reward processing, which is consistent with the idea that curiosity reduction is rewarding. This idea is consistent with previous behavioral findings that people actively prefer to view the clear version of a preceding blurred picture over viewing an unrelated clear picture (Nicki, 1970). Other work has shown that people have a similar preference for exploring perceptually novel over familiar stimuli, a tendency that is also associated with striatal activation (Wittmann et al., 2008). In the reinforcement-learning literature, this bias towards the exploration of uncertain or novel options is captured by the concept of an "exploration bonus" that is assigned to uncertain or novel stimuli to promote their exploration (Kakade and Dayan, 2002).

The relief of perceptual curiosity was also associated with enhanced incidental memory, and with increased hippocampal activation, a plausible neural substrate underlying the behavioral memory effect. The finding that curiosity reduction leads to enhanced memory suggests that inducing people's curiosity before presenting them with teaching material (e.g., by asking people to guess the meaning of foreign words before showing them the translations) can facilitate learning.

Because the AIC and ACC are the two brain regions that are typically activated by aversive conditions, and the striatum plays a key role in reward processing, our fMRI results are consistent with our predictions that curiosity is an aversive state whose termination is rewarding. However, it is important to note that the AIC/ACC and the striatum are also involved in cognitive processes that are unrelated to aversive states and rewards, respectively. Due to the non-specificity of the function of these brain areas, it is possible that the activations we found reflected processes other than those predicted by Berlyne's theory; hence our findings do not provide persuasive evidence for that theory. Indeed, our method of inferring the presence of particular cognitive/affective processes from the activation of particular brain regions is an example of "reverse inference" (Poldrack, 2006). Although

this method is not deductively valid, it can provide interesting hypotheses about the underlying mechanisms of relatively unexplored conditions, such as curiosity, which can then be tested in subsequent studies. Thus, our study should be seen as a first demonstration of the neural correlates of perceptual curiosity, of which the results are consistent with but do not provide unequivocal evidence for Berlyne's theory. Future studies testing more specific predictions are necessary to either confirm or reject our interpretation of the curiosity-related brain activation.

Curiosity is a multifaceted construct, and several different types of curiosity can be distinguished. One important distinction is the difference between *perceptual* and *epistemic* curiosity. Perceptual curiosity is aroused by novel, strange or ambiguous stimuli, whereas epistemic curiosity refers to the desire for knowledge or intellectual information which applies mainly to humans (Berlyne, 1954). Another, orthogonal, distinction can be made between *specific* and *diversive* curiosity, referring to the desire for a particular piece of information versus the more general stimulation-seeking motive that is closely related to boredom (Berlyne, 1960; see Litman, 2008, for a related distinction). The type of curiosity that we investigated in the present study can be referred to as specific perceptual curiosity, one of the most basic types of curiosity that applies to both animals and humans. The neural substrates of specific epistemic curiosity evoked by trivia questions was investigated recently (Kang et al., 2009). Kang et al. found that questions that were rated as more puzzling were associated with stronger activation in regions of the caudate. However, since the questions were always followed by their correct answers, it was unclear whether this activation reflected uncertainty about the correct answer, feedback anticipation, or a combination of the two. In our study, the uncertainty induced by blurred pictures was often not relieved, which allowed examination of the neural correlates of pure curiosity unconfounded by the anticipation of uncertainty-reducing feedback. In addition, by comparing conditions in which the second picture did vs. did not reduce perceptual uncertainty, we could separately isolate the neural correlates of the relief of curiosity.

We did not ask participants to rate their curiosity on each trial since we were concerned that this would confound the brain activation reflecting their natural curiosity. Therefore, a limitation of our study is that we could not take into account trial-to-trial variation in experienced curiosity. In addition, it must be noted that the blurred pictures in our study were associated with both perceptual uncertainty and outcome uncertainty, since the identity of the blurred image was revealed in only half of the trials. Thus, participants may have experienced uncertainty not only about the identity of the image, but also about whether or not the identity of the image would be resolved. Both forms of uncertainty are likely to increase overall curiosity, but future studies in which these two forms of uncertainty are manipulated independently are needed to assess their respective contributions to brain activation. Finally, it is likely that curiosity reduction through passive exposure to uncertainty-reducing stimuli, as examined in the present study, differs from curiosity reduction that is achieved through active exploration. A recent study showed that hippocampus activation was stronger when people had volitional control over the visual exploration of pictures in a visual-learning task than when they received exactly the same visual information in a passive condition (Voss et al., 2011). This suggests that the hippocampus activation associated with uncertainty reduction that we found in the present study would have been even stronger if participants would have had the opportunity to actively control the exploration of uncertainty-inducing stimuli.

To conclude, our results provide the first demonstration of the neural correlates of human perceptual curiosity, and are consistent with the assumptions of Berlyne's theory that perceptual curiosity evokes an aversive state of increased arousal, whose termination is rewarding and promotes incidental memory. Because curiosity plays a key role in many aspects of human behavior, a better understanding of the psychological and neurobiological basis of curiosity may have considerable practical implications for various societal objectives. Together with previous behavioral findings (Berlyne and Normore, 1972), our results suggest that inventing ways to arouse people's curiosity could contribute to the optimization of educational systems and advertising strategies, and may promote scientific discovery.

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Preventive role of social interaction for cocaine conditioned place preference: correlation with FosB/DeltaFosB and pCREB expression in rat mesocorticolimbic areas

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†Author Contributions:
Rana El Rawas, Gerald Zernig, Alois Saria and Michael Fritz designed the experiments. Sabine Klement and Michael Fritz performed the behavioral study. Rana El Rawas performed the immunohistochemistry. Ahmad Salti performed the qRT-PCR. Rana El Rawas and Ahmad Salti analyzed the data. Rana El Rawas wrote the paper. Gerald Zernig and Alois Saria have critically reviewed the contents of the paper and provided instrumental suggestions. Alois Saria and Georg Dechant provided the infrastructure required to perform the experiments.

The worsening of drug abuse by drug-associated social interaction is a well-studied phenomenon. In contrast, the molecular mechanisms of the beneficial effect of social interaction, if offered as a mutually exclusive choice to drugs of abuse, are under-investigated. In a rat place preference conditioning (CPP) paradigm, four 15 min episodes of social interaction with a gender- and weight-matched male early-adult conspecific inhibited cocaine-induced reinstatement of cocaine CPP, a model of relapse. These protective effects of social interaction were paralleled by a reduced activation, as assessed by Zif268 expression, in brain areas known to play pivotal roles in drug-seeking behavior. Here we show that social interaction during extinction of cocaine CPP also reduced cocaine-CPP-stimulated FosB expression in the nucleus accumbens shell and core. In addition, social interaction during cocaine CPP extinction increased pCREB (cAMP response element binding protein) expression in the nucleus accumbens shell and the cingulate cortex area 1 (Cg1). Our results show that FosB and pCREB may be implicated in the protective effect of social interaction against cocaine-induced reinstatement of CPP. Thus, social interaction, if offered in a context that is clearly distinct from the previously drug-associated one, may profoundly inhibit relapse to cocaine addiction.

Keywords: cocaine, conditioned place preference, social interaction, FosB/ΔFosB, pCREB, relapse, substance-use disorder

INTRODUCTION

Drug dependence is a multifactorial disorder resulting from an interaction between genetic, social, and environmental factors (Kreek et al., 2005; Enoch, 2006). There is compelling evidence that social experiences modify vulnerability to reinstatement, acting as prevention or risk factors in the development of drug addiction (Swadi, 1999). Using animal models, several studies have investigated how social interaction, if made available together with a drug of abuse, may worsen drug abuse and substance dependence (Gauvin et al., 1994; Thiel et al., 2008, 2009; Ribeiro Do Couto et al., 2009). However, there is little research on how social interaction, if offered as an alternative to drug consumption, affects neural circuits involved in drug reinforcement and substance dependence.

We have recently shown that only four social interaction episodes (15 min each) with a male early-adult conspecific as an alternative (i.e., non-drug-associated) stimulus completely reversed conditioned place preference (CPP) for cocaine (15mg/kg i.p.) and were even able to inhibit cocaine-induced reinstatement of cocaine CPP (Fritz et al., 2011a). The behavioral effects of social interaction were paralleled by effects on the brain circuitry known to be involved in drug reinforcement and reward. Social interaction during extinction of cocaine CPP reversed cocaine CPP-reinstatement-associated Zif268 expression in the nucleus accumbens shell, the central and basolateral amygdala, and the ventral tegmental area (Fritz et al., 2011a). We have also shown that the sigma1 receptor antagonist BD1047 enhances reversal of conditioned place preference from cocaine to social

interaction (Fritz et al., 2011b). These findings suggest that social interaction, if offered in a context that is clearly distinct from the previously drug-associated one, may profoundly decrease the incentive salience of drug-associated contextual stimuli.

Drugs of abuse are known to cause several neuroadaptations in dopaminergic as well in other neurotransmitter systems. One of these adaptations is an altered expression of transcription factors that engender changes in gene expression and may possibly lead to alterations in sensitivity to drugs of abuse (Hyman and Malenka, 2001; Nestler et al., 2001). One of these transcription factors is ΔFosB. In contrast to the other Fos proteins, ΔFosB is induced to only a small degree in response to acute drug administration but because of its unique stability, after repeated drug administration, ΔFosB gradually accumulates in the striatum and stays elevated for weeks or months after discontinuation of drug exposure (Hope et al., 1994; Chen et al., 1995; Moratalla et al., 1996; Hiroi et al., 1997). Thus, it has been hypothesized that ΔFosB functions as a sustained molecular switch that mediates some of the more persistent adaptations of the brain that underlie addiction (McClung et al., 2004). The first study of *fosB* KO mice found that these mice develop a robust CPP to a lower dose of cocaine compared with wild type mice (Hiroi et al., 1997). However, it has been found that over-expression of ΔFosB leads to enhanced rewarding and reinforcing effects of drugs of abuse. Indeed, over-expression of ΔFosB in the striatum resulted in maximal CPP to a low dose of cocaine (Kelz et al., 1999) and facilitated the acquisition of cocaine self-administration at low doses (Colby et al., 2003). Another transcription factor implicated in the effects of drugs of abuse is CREB (cAMP response element binding protein). CREB is a constitutively expressed transcription factor the activity of which is tightly regulated by its phosphorylation at serine 133 (Lonze and Ginty, 2002). In the nucleus accumbens, it has been shown that increased CREB activity decreases the rewarding effects of cocaine and morphine in the CPP paradigm (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002; Dinieri et al., 2009). Furthermore, CREB activity in brain regions other than the nucleus accumbens seems to regulate the rewarding effects of drugs of abuse as well. Accordingly, it has been found that over-expression of CREB in the rostral ventral tegmental area enhanced the development of cocaine- and morphine-induced CPP (Olson et al., 2005).

Given that social interaction during extinction was able to prevent reinstatement of cocaine CPP and to reduce activation in reward-related brain areas as assessed by Zif268 protein expression (Fritz et al., 2011a), we aimed to expand our findings to explore the involvement of other transcription factors in social interaction preventive effect. Therefore, in the present study we investigated the expression of FosB/ΔFosB and pCREB proteins using immunohistochemistry in mesocorticolimbic areas in brains of (1) naïve rats, (2) rats that underwent cocaine CPP followed by saline extinction, and (3) rats that underwent cocaine CPP followed by social interaction during saline extinction (counterconditioning).

MATERIALS AND METHODS

ANIMALS

Male Sprague–Dawley rats (150–250) g, corresponding to an age of (6–8 weeks) which can be considered at early adulthood (Spear, 2000), were obtained from the Research Institute of Laboratory Animal Breeding of the Medical University Vienna (Himberg, Austria) and were group-housed (six rats per cage) at 24°C. The animals received *ad libitum* access to tap water and pellet chow. A 12 h light/dark cycle, with lights on from 0800 h to 2000 h, was maintained. Single housing commenced at the start of the behavioral experiment and continued throughout the experiment which was conducted during the light period of the cycle. The animals used in this study were cared for in accordance with the guidelines of the National Institutes of Health Animal Care and Use Program and the NIDA-IRP Animal Program, and the present experiments were approved by the Austrian National Animal Experiment Ethics Committee.

PLACE CONDITIONING APPARATUS

Conditioning was conducted in a homemade three chamber apparatus (63 cm wide × 33 cm deep × 30 cm high) made of plywood panels covered with plastic film. The middle (neutral) compartment (10 × 30 × 30 cm) had gray walls and a gray floor. The two conditioning chambers (25 × 30 × 30 cm) had either black walls with two vertical white stripes (5 × 30 cm) on each side and a stainless steel floor with 20 holes (diameter 0.5 cm) or black walls with five horizontal white stripes (3 × 25 cm) and a stainless steel floor with 15 slits (5 × 0.5 cm). Time spent in each compartment was taken with hand timers. If the added-up times for all three compartments were less than the total 900 s of the test session, the missing time was distributed equally among the three chambers to avoid any bias. After every single rat, the apparatus was cleaned with a 70% camphorated ethanol solution.

PLACE CONDITIONING PROCEDURE

Training

Conditioned place preference was conducted as described previously (Fritz et al., 2011a). All experiments were performed by two individual experimenters using white light (15 Watt) and radio-generated white noise. During the pre-training test, rats were allowed to move freely between the three chambers for 15 min (900 s). Pre-test bias for the chambers was controlled for at the experimental group level by comparing means and SEMs of the times spent in any of chambers subsequently used for cocaine or saline pairing. If SEMs were overlapping, the group was advanced to CPP training. SEMs were chosen because they represent the numerically smallest measure of variance, thus yielding the most conservative group pre-test bias criterion. To further control for a pre-test bias at the individual level that may have escaped our group pre-test bias criterion, we chose the following arbitrary criterion: if any individual animal remained >349 s in one of the outer two compartments, cocaine was paired with the initially non-preferred side. This happened in about 10–15% of the animals. We have no indication that the application of these two criteria generated an "unbiased" vs. a "biased" population, which would have been evident as a bimodal distribution in pre-test times. Cocaine (hydrochloride salt, a gift from the National Institute on Drugs of Abuse to Gerald Zernig, corresponding to 15 mg/kg pure cocaine base in a volume of 1 ml/kg saline) or saline was injected intraperitoneally (i.p.) immediately before

placing the rat into the closed dedicated chamber (cocaine- vs. saline allocation counterbalanced within-group). If a compartment was paired with social interaction during CPP training, each rat received an i.p. injection of saline and was placed in the compartment to allow for social interaction with a conspecific of the same weight and gender (male) during the whole 15 min conditioning session. Each rat was assigned a different partner, which stayed the same for the whole duration of the experiment. Gross observation indicated that only "agonistic" (i.e., "friendly") social interaction, i.e., touching, crawling under, and grooming occurred, whereas "antagonistic" (i.e., threatening, boxing, fighting, biting, etc.) behavior was not observed during the training sessions. Both animals remained singly housed. Place preference was conditioned using an alternate-day design of four once-daily 15 min training sessions for each condition. The CPP test was performed 24 h after the last conditioning trial by placing the rat in the middle (neutral) compartment of the CPP apparatus and allowing it to move freely between the three compartments for 15 min. Only animals that had established cocaine CPP were used in the extinction and reinstatement experiments.

Extinction of cocaine CPP: effect of social interaction

After cocaine CPP had been established, subjects were divided into two groups. One group received i.p. saline injections immediately before being put into the former cocaine-paired chamber as well as into the previously saline-paired chamber for one extinction session each (during a total of two consecutive days). The second group received an i.p. saline injection immediately before being placed into the previously cocaine-paired chamber for 15 min on one day but, in contrast to the previous group, was also given the opportunity to have social interaction in the previously saline-paired chamber with a conspecific on the other day. On the third day, animals were tested for CPP (15 min; T1). The three-day cycle of training-training-test was repeated three more times (T2, T3, T4).

Cocaine-induced reinstatement of cocaine CPP: effect of social interaction

Twenty-four hours after the last extinction training, rats were administered a single i.p. cocaine injection in the previously cocaine-paired compartment. Another 24 h later, all rats were tested for CPP in a drug-free state.

Immunohistochemistry

FosB/ΔFosB and pCREB immunohistochemistry were per formed by an experimenter who was blind to treatment conditions. Two hours after the start of the cocaine reinstatement test i.e., 26 h after the last cocaine exposure, rats were deeply anesthetized using isoflurane and intracardially perfused with 0.1 M phosphate buffer followed by 4% paraformaldehyde (PFA) dissolved in 0.1 M phosphate buffered saline (PBS; pH 7.4). Brains were then removed and post-fixed in 4% PFA overnight, then stored in 30% sucrose at 4°C until the brain sank, and then at −80°C until sectioning. All serial brain sections (40 μm) were cut using a Cryostat (Leica). Sections were stored in an assorter buffer (Tris buffer 0.25 M, NaN_3 10%) at 4°C until processed for immunolabeling.

Free-floating sections from rats in different groups (naïve rats, cocaine CPP + saline extinction, and cocaine CPP + social interaction extinction) were processed simultaneously for FosB/ΔFosB or pCREB protein expression. Sections were washed in PBS 0.1 M and incubated for 30 min in 0.3% hydrogen peroxide/PBS 0.1 M. Then they were washed in PBS 0.1 M and incubated for 1 h in 0.3% Triton X-100 in PBS 0.1 M containing 3% normal goat serum. Subsequently sections were incubated for 24 h with N terminal anti-FosB rabbit primary antibody that recognizes both FosB and ΔFosB proteins at room temperature (1:5000, Santa Cruz Biotechnology) or pCREB rabbit primary antibody at 4°C (1:2000, Millipore) containing 0.3% Triton X-100, and 1% normal goat serum in PBS 0.1 M. Sections were washed in PBS and incubated for 1 h and 30 min in PBS containing biotinylated goat anti-rabbit antibody IgG (1:200, Vector Laboratories), 0.3% Triton X-100, and 1% normal goat serum. Afterward the tissue was given additional washes in PBS 0.1 M and incubated for 90 min in avidin-biotinylated horseradish peroxidase complex (ABC Elite kit, Vector Laboratories) diluted in PBS 0.1 M. Then, sections were washed in PBS 0.1 M followed by an incubation in 3,3-diaminobenzidine tetrahydrochloride (DAB tablets, Sigma). The reaction was terminated by rinsing the tissue in PBS 0.1 M. Finally, sections were then mounted onto gelatin-coated slides, dried, and dehydrated before coverslipping.

Brain sections were scanned using a Zeiss optical microscope set at 20× magnification equipped with a camera (Axioplan 2 Imaging) interfaced to a PC. Immunoreactive nuclei were counted by an observer who was blind to treatment conditions using Metamorph imaging software. Each rat contributed the averaged value of two sections per brain area to the group mean. Immunoreactivity was expressed as stained nuclei per mm^2 of the respective brain regions (**Figure 5**) identified according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007): the prelimbic (PrL) and infralimbic (IL) cortex, the anterior cingulate cortex areas Cg1 and Cg2, the dorsal striatum (caudate putamen, CPu), the nucleus accumbens core (AcbC) and shell (AcbSh) subregions, the central (Ce) and basolateral (BLA) amygdala, and the ventral tegmental area.

Quantitative real time polymerase chain reaction (qRT-PCR)

All qRT-PCR experiments were performed by an experimenter who was blind to treatment conditions. Forty-five minutes after exposure to the cocaine-associated chamber, i.e., 30 min after the end of the reinstatement test, brains were rapidly removed and immediately frozen in −40°C isopentane on dry ice. Brain regions were removed by freehand using a sterile blade while viewing sections using magnifying goggles from thaw-mounted coronal 200 μm sections at −15°C in a cryostat. The bregma coordinates according to the atlas of Paxinos and Watson (2007 ed.) was 2.52–0.84 mm for the Acb.

Total RNA was isolated from the dissected brain regions using Trizol (Invitrogen) according to the manufacturer's recommendations. To avoid contamination with genomic DNA, total RNA was treated with DNase (2 U/μL) using the TURBO DNA-free Kit (Ambion). RNA was reverse transcribed in the presence of random hexamer primers and MultiScribe Reverse Transcriptase

(50 U/μL) in a total volume of 20 μl employing the high-capacity cDNA reverse transcription kit with RNase Inhibitor (Applied Biosystems).

After dilution with 80 μl of water, 3 μl of the diluted cDNA was used as a template for amplification (duplicates) with AB Fast SYBR Green mastermix (Applied Biosystems). RT-PCR quantification was performed on a 7500 Fast Real Time PCR system (Applied Biosystems) using the following cycle settings: 20 s 95°C, 40 cycles of 95°C for 3 s, and 60°C for 30 s. All PCR primers were designed using PrimerSelect 5.05 software (DNASTAR). Ct and ΔCt values were calculated by using the 7500 software v2.0.1 and GAPDH as a reference gene.

Primer sequences used in the quantitative real time polymerase chain reaction:

	Genbank	Fwd	Rev
Cdk5	NM_080885	CTGTTGCAGA ACCTGTTGAAG	CCAGGGTCA GAGAGTCTAC
Nfkb	XM_342346	CGATCTGTATC AGACACCTTTG	TGCCTTGCTGT TCTTGAGTAG

Data analysis

All results are presented as group means ± SEM. Behavioral results were analyzed using One-Way or Two-Way repeated-measures analyses of variance (ANOVA). One-Way repeated-measures ANOVA models always included the within-subjects factor treatment (compartment), two-factor repeated-measures ANOVA models included the factors treatment and time. Statistical significance of both main effects and interactions was tested. Moreover, *post-hoc* comparisons between individual factor levels (e.g., cocaine vs. saline) were performed using Tukey's test. Differences in protein expression in individual brain regions and mRNA expression were performed using One-Way ANOVA. *Post-hoc* comparison of individual treatments was done in the same way as above. All statistical tests were performed at a 0.05 level of significance. All statistical analyses were conducted with stat view program.

RESULTS

EXTINCTION AND REINSTATEMENT OF COCAINE CPP: EFFECT OF SOCIAL INTERACTION ON BEHAVIOR

Animals acquired robust conditioned place preference to cocaine ($P < 0.001$; **Figure 1**). Cocaine CPP could be extinguished over four extinction cycles that were administered over a period of 12 days (**Figure 1A**; $n = 17$). Two-Way repeated-measures ANOVA with treatment and time (CPP to T4) as within-subject factors [of CPP tests T1–T4] performed during extinction revealed a significant treatment effect (previously cocaine-associated chamber vs. previously saline-paired chamber; $P = 0.023$), a significant time effect ($P = 0.002$) and a significant time-by-treatment interaction ($P < 0.001$). For T1 [Time in compartments (sec): drug-paired: 377 ± 34; neutral: 262 ± 14; saline-paired: 262 ± 29], a near-significant preference for the initially cocaine-associated compartment over the saline-associated compartment was found ($P = 0.054$), whereas tests T2 [Time in compartments (sec): drug-paired: 316 ± 22; neutral: 286 ± 13; saline-paired: 280 ± 23], T3 [Time in compartments (sec): drug-paired: 316 ± 26; neutral: 268 ± 16; saline-paired: 316 ± 22], and T4 (**Figure 1A**) did not yield any significant preference for the cocaine-associated compartment any more ($P > 0.1$).

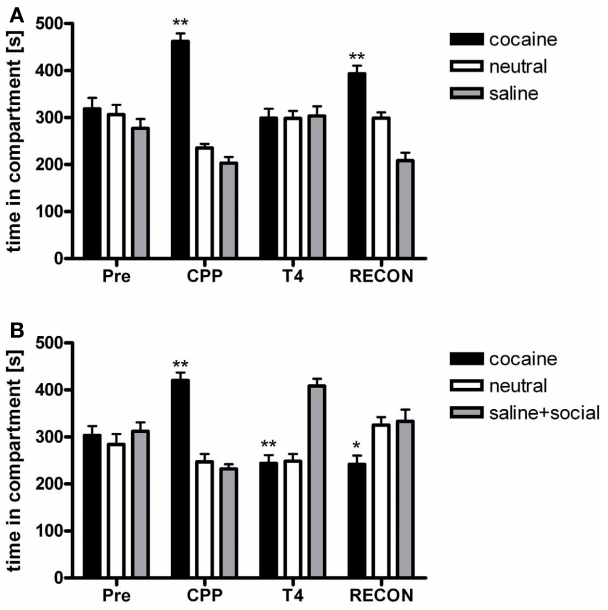

FIGURE 1 | Social interaction during extinction reverses cocaine CPP and inhibits reinstatement of cocaine CPP. PRE, preconditioning test; CPP, conditioned place preference test; T4, conditioned place preference test during extinction in absence (panel A) or presence (panel B) of social interaction made available in the initially saline-associated compartment; RECON, reinstatement test. Shown is time spent in the cocaine, neutral, or saline-associated compartment (group means ± SEM). $^*P < 0.05$ and $^{**}P < 0.01$ compared to the time spent in the saline-associated chamber.

If social interaction was made available in the previously saline-paired chamber during extinction only once (i.e., during the first extinction cycle), conditioned place preference for cocaine was no longer observed during the subsequent CPP test ($n = 18$; T1). In the subsequent extinction (counterconditioning) cycles, pronounced preference for the social interaction-associated chamber developed (**Figure 1B**, T4), leading to a reversal of CPP from cocaine to social interaction. Two-Way repeated-measures ANOVA showed a non-significant treatment main effect (social interaction vs. saline, $P > 0.1$) and a non-significant time effect ($P > 0.1$), but a highly significant treatment × time interaction ($P < 0.001$). The non-significance of the main effects of the factors time and treatment is a consequence of the reversal of CPP described above. A significant preference of the saline-social interaction chamber over the initially cocaine-associated chamber was observed at CPP ($P < 0.001$), T3 [Time in compartments (sec): drug-paired: 242 ± 20; neutral: 243 ± 14; saline + social interaction-paired: 416 ± 21, $P < 0.001$] and T4 (**Figure 1B**, $P < 0.001$), whereas no significant difference between the times spent in the respective chambers was seen at T1 [Time in compartments (sec): drug-paired: 341 ± 37; neutral: 245 ± 20; saline + social interaction-paired: 315 ± 29] and T2 [Time in compartments (sec): drug-paired:

284 ± 26; neutral: 273 ± 19; saline + social interaction-paired: 342 ± 25].

If cocaine CPP extinction (saline extinction) was followed by one cocaine-chamber pairing session, fully extinguished cocaine CPP was reinstated (**Figure 1A**) [repeated-measures ANOVA, treatment effect (cocaine vs. neutral vs. saline), $P < 0.001$; cocaine vs. saline, $P < 0.001$]. However, if social interaction was available in the previously saline-paired chamber during cocaine CPP extinction, reinstatement of cocaine CPP was not only fully prevented but preference for the social interaction-paired compartment remained (**Figure 1B**) [repeated-measures ANOVA, treatment effect (social interaction vs. neutral vs. cocaine), $P = 0.037$; cocaine vs. social interaction, $P = 0.045$].

REINSTATEMENT OF COCAINE CPP: EFFECT OF SOCIAL INTERACTION ON THE EXPRESSION OF THE FosB/ΔFosB TRANSCRIPTION FACTORS

Two hours after the cocaine reinstatement test performed in the drug-free state, i.e., 26 h after the last cocaine exposure, we processed the rats' brain for immunohistochemistry to investigate changes in FosB/ΔFosB expression in brain areas implicated in drug reinforcement. FosB/ΔFosB expression was significantly different between treatments (naive $n = 9$, cocaine CPP followed by saline extinction $n = 7$, and cocaine CPP followed by social interaction $n = 8$) in the following brain areas (**Figure 2**): CPu [One-Way ANOVA, treatment effect: $P < 0.0001$], AcbSh [One-Way ANOVA, treatment effect: $P < 0.0001$], AcbC [One-Way ANOVA, treatment effect: $P < 0.001$], and BLA [One-Way ANOVA, treatment effect: $P < 0.001$]. Cocaine CPP in the absence of social interaction as an alternative (i.e., non-drug) stimulus during extinction significantly increased FosB/ΔFosB expression as compared to experiment-naïve animals (**Figure 2**) in the CPu ($P < 0.01$), AcbSh ($P < 0.01$), AcbC ($P < 0.01$), and the BLA ($P < 0.01$). While social interaction during extinction of cocaine CPP increased FosB/ΔFosB expression in the CPu ($P < 0.01$), AcbSh ($P < 0.01$), and the BLA ($P < 0.01$) in comparison to naive rats, it decreased cocaine CPP-reinstatement-associated FosB/ΔFosB expression (**Figure 2**) in the AcbSh ($P < 0.01$) and the AcbC ($P < 0.05$).

As there is no antibody available to distinguish between FosB and the different isoforms of the truncated FosB protein ΔFosB and as it is not well known if exposure to reward-associated conditioned stimuli causes FosB expression (Harris et al., 2007),

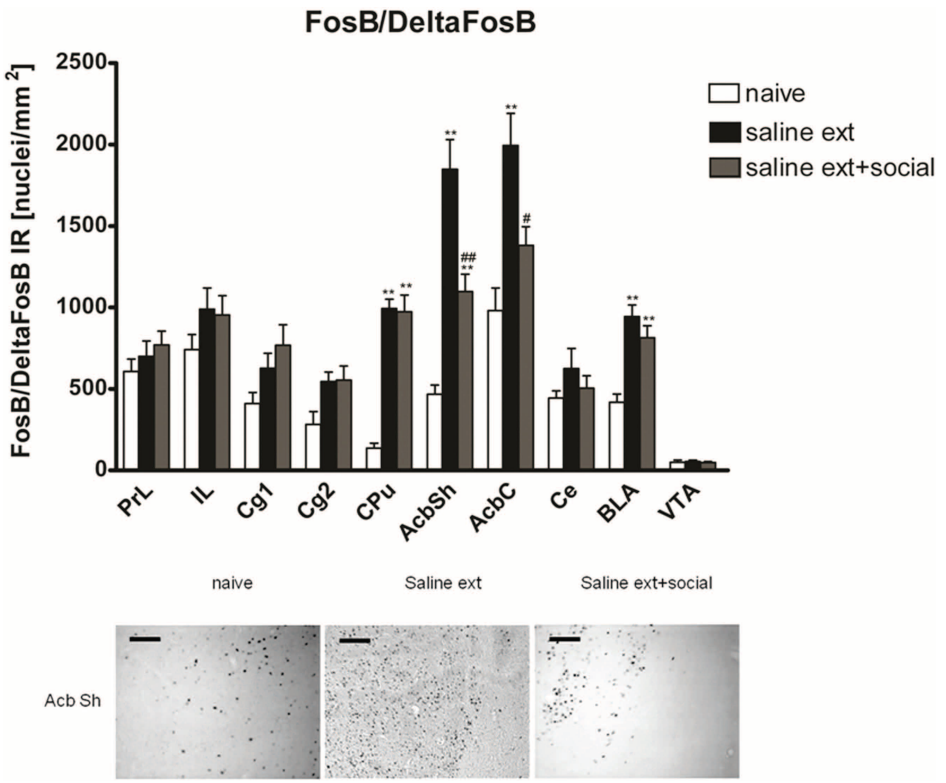

FIGURE 2 | Social interaction decreases cocaine CPP reinstatement-associated FosB/ΔFosB expression in nucleus accumbens shell and core. Shown are FosB/ΔFosB positive immuno-reactive nuclei per mm^2 of the respective brain area. Shown are group means ± SEM. $^{**}P < 0.01$ compared to naive group; $\#P < 0.05$ and $\#\#P < 0.01$ compared to saline extinction group. In the dorsal striatum (CPu) and the basolateral amygdala (BLA), priming with cocaine after saline or saline + social extinction increased FosB/ΔFosB expression in comparison to naive rats ($^{**}P < 0.01$). Brain area abbreviations follow the nomenclature of Paxinos and Watson (Paxinos and Watson, 2007). In the low panel, FosB-like immuno-reactive nuclei are given for the nucleus accumbens shell (AcbSh). Sections were taken from completely experiment-naïve rats (naïve), or from rats that underwent extinction before the cocaine reinstatement test in absence (sal ext) or presence of a weight-matched male conspecific (sal ext + social). Scale bar: 100 μm.

we proceeded as follows to investigate if the reduced expression of FosB/ΔFosB in the Acb (AcbSh and AcbC) of rats that express CPP and undergo social interaction is due to FosB or ΔFosB:

(1) We assessed the expression of cdk5 and nfkb, targets of ΔFosB (Nestler, 2008) in the Acb of rats that underwent extinction of cocaine CPP in presence or absence of social interaction by qRT-PCR. Our results show that cocaine treatment used in this study [four injections of cocaine (15 mg/kg) during conditioning + one injection (15 mg/kg) to induce reinstatement] was not sufficient to induce the expression of ΔFosB targets cdk5 and nfkb. In addition, social interaction did not change expression of cdk5 [One-Way ANOVA, treatment effect: $p > 0.05$; naive ($n = 4$): 5.4 ± 0.4; saline extinction group ($n = 5$): 5.7 ± 0.4; social interaction group ($n = 6$): 5.3 ± 0.4] or nfkb [One-Way ANOVA, treatment effect: $p > 0.05$; naive ($n = 4$): 6.9 ± 0.1; saline extinction group ($n = 5$): 6.8 ± 0.2; social interaction group ($n = 6$): 6.7 ± 0.2].

(2) We trained another group of rats for cocaine CPP and extinguished cocaine CPP in presence or absence of social interaction. These rats were then administered an i.p. cocaine injection but were not exposed to the cocaine-associated CPP compartment (without the CPP reinstatement test done 24 h after the last cocaine injection) to eliminate the possibility of expression of FosB as an immediate early gene by the cues associated with cocaine CPP. Thus, the FosB/ΔFosB expression that we analyzed in this experiment 26 h after the last injection of cocaine should, in all likelihood, represent almost solely ΔFosB (Chen et al., 1997; Nestler, 2004; Ulery et al., 2006; Perrotti et al., 2008). The use of these animals enabled us not only to investigate the expression of ΔFosB protein alone but also consider any possible involvement of the full length FosB protein in social interaction protective effect by comparing ΔFosB expression to FosB/ΔFosB expression both analyzed 26 h after the last cocaine exposure. Using immunohistochemistry, we could investigate the shell and core subregions of the Acb in addition to other brain areas involved in drug rewarding effects. ΔFosB expression was significantly different between treatments (naive $n = 9$, cocaine CPP followed by saline extinction $n = 5$, and cocaine CPP followed by social interaction during saline extinction $n = 6$) in the following brain areas (**Figure 3**): CPu [One-Way ANOVA, treatment effect: $P < 0.0001$], AcbSh [One-Way ANOVA, treatment effect: $P < 0.001$], and AcbC [One-Way ANOVA, treatment effect: $P < 0.05$]. Cocaine CPP in the absence or presence of alternate social interaction during extinction increased similarly ΔFosB expression as compared to experiment-naïve animals (**Figure 3**) in the CPu ($P < 0.01$), AcbSh ($P < 0.01$), AcbC ($P < 0.05$).

The reduction of FosB/ΔFosB (**Figure 2**) and not ΔFosB (**Figure 3**) expression (both assessed 26 h after the last cocaine injection) in the AcbSh and AcbC after social interaction suggests that FosB is the transcription factor involved in the protective effect of social interaction.

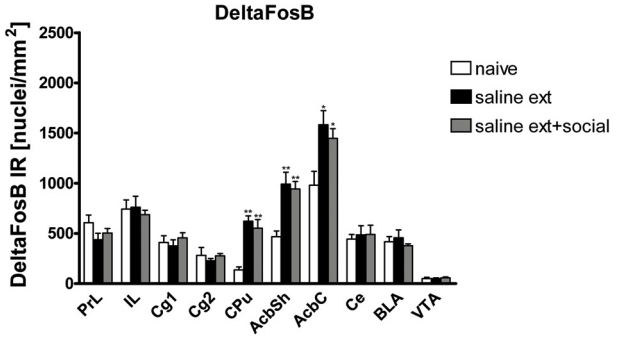

FIGURE 3 | Cocaine CPP reinstatement-associated ΔFosB expression is increased similarly in the dorsal striatum (CPu), nucleus accumbens shell (AcbSh), and core (AcbC) of rats that underwent extinction before the cocaine priming in absence (sal ext) or presence of social interaction (sal ext + social). Shown are ΔFosB positive immuno-reactive nuclei per mm^2 of the respective brain area. Shown are group means ± SEM. $*P < 0.05$ and $**P < 0.01$ compared to naive group. Brain area abbreviations follow the nomenclature of Paxinos and Watson (Paxinos and Watson, 2007).

REINSTATEMENT OF COCAINE CPP: EFFECT OF SOCIAL INTERACTION ON THE EXPRESSION OF pCREB TRANSCRIPTION FACTOR

Given that CREB and ΔFosB usually produce opposite effects on either the expression of numerous genes or a number of behavioral phenotypes, we investigated pCREB expression in brain areas involved in drug rewarding effects of rats that did not undergo the reinstatement test (same animals used to assess ΔFosB expression) using immunohistochemistry. pCREB expression was significantly different between treatments (naive $n = 7$, cocaine CPP followed by saline extinction $n = 5$, and cocaine CPP followed by social interaction during saline extinction $n = 6$) in the following brain areas (**Figure 4**): Cg1 [One-Way ANOVA, treatment effect: $P < 0.05$] and AcbSh [One-Way ANOVA, treatment effect: $P < 0.05$]. Social interaction during extinction of cocaine CPP increased pCREB expression in the Cg1 ($P < 0.01$) and AcbSh ($P < 0.05$) as compared to rats that undergo cocaine CPP in the absence of alternate social interaction during extinction (**Figure 4**).

DISCUSSION

We have previously shown that social interaction during extinction of cocaine CPP reversed cocaine CPP-reinstatement-associated Zif268 expression in the nucleus accumbens shell, the central and basolateral amygdala, and the VTA (Fritz et al., 2011a). In the present study, we found that social interaction during extinction of cocaine CPP also decreased FosB expression in the nucleus AcbC and shell, and increased pCREB expression in the nucleus accumbens shell and Cg1 area of the cingulate cortex associated with cocaine-induced reinstatement of CPP.

Studies with gene modified animals and viral vectors have shown that an over-expression of ΔFosB in certain brain regions such as the nucleus accumbens creates a behavioral phenotype

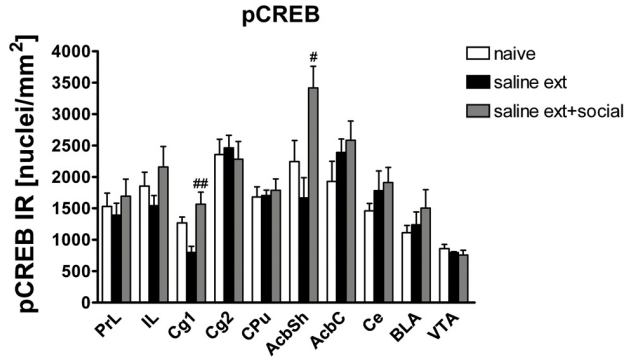

FIGURE 4 | Social interaction increases cocaine CPP reinstatement-associated pCREB expression in nucleus accumbens shell (AcbSh) and cingulated cortex (Cg1). Shown are pCREB positive immune-reactive nuclei per mm^2 of the respective brain area. Shown are group means ± SEM. #$P < 0.05$ and ##$P < 0.01$ compared to saline extinction group. Brain area abbreviations follow the nomenclature of Paxinos and Watson (Paxinos and Watson, 2007).

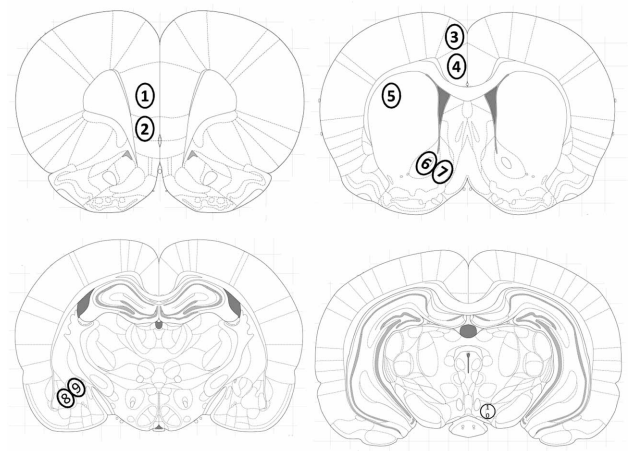

FIGURE 5 | Location of investigated brain areas. Brain areas were identified according to the atlas of Paxinos and Watson (Paxinos and Watson, 2007): the (1) prelimbic (PrL) and (2) infralimbic (IL) cortex, the cingulate cortex areas (3) Cg1 and (4) Cg2, (5) dorsolateral striatum (caudate putamen, CPu), the (6) nucleus accumbens core (AcbC) and (7) shell (AcbSh) subregions, (8) basolateral (BLA) and (9) central (Ce) amygdala, and (10) the ventral tegmental area (VTA).

with increased sensitivity to drugs of abuse (Kelz et al., 1999; Colby et al., 2003; Peakman et al., 2003; Zachariou et al., 2006). Given that social interaction was able to prevent reinstatement of cocaine CPP, we expected that rats which had the opportunity to interact socially during cocaine CPP extinction would show decreased ΔFosB expression in the nucleus accumbens. Social interaction significantly decreased cocaine CPP-reinstatement-associated FosB/ΔFosB expression in the nucleus accumbens shell and core. However, as it is not well known if exposure to cues conditioned to rewards causes FosB expression (Harris et al., 2007), we cannot identify which transcription factor is implicated in the protective effect of social interaction. We first investigated ΔFosB targets cdk5 and nfkb mRNA expression in the nucleus accumbens (Nestler, 2008) of naïve and cocaine CPP-expressing rats followed by extinction in presence or absence of social interaction. Unexpectedly, we found no difference between these three groups of rats in the expression of either target of ΔFosB. These results suggest that (1) ΔFosB may not be the transcription factor implicated in the protective effect of social interaction or (2) As cdk5 mRNA expression depends highly on the dose of cocaine given, the protocol of injection (sensitization vs. desensitization), species (mice vs. rats), cocaine self-administration vs. injection (Wedzony et al., 2005); cocaine injections that the rat received during this study (four injections of 15 mg/kg each for CPP training every second day and one injection of 15 mg/kg to induce reinstatement) were not sufficient to produce changes in the targets of ΔFosB. While most of the studies investigating cdk5 mRNA expression are based on subsequent viral-mediated ΔFosB over-expression in mice (Bibb et al., 2001), the same group has also found a difference in cdk5 mRNA expression in the CPu and the nucleus accumbens of rats that received chronic injections of cocaine at the dose of 20 mg/kg i.p./day for eight days which represents a higher dose of cocaine and a different regimen of administration in comparison to the present study (Bibb et al., 2001). In order to explore separately ΔFoB expression in the nucleus accumbens subregions shell and core, we performed immunohistochemistry in rats that were treated exactly the same as before but without being exposed to the cues associated to cocaine CPP to eliminate possibilities of FosB induction. We found that cocaine CPP in absence or presence of social interaction as the non-drug (alternative) stimulus during cocaine CPP extinction increased ΔFosB expression to the same degree as compared to experiment-naïve animals in both subregions of the nucleus accumbens. Consequently, these results suggest that FosB could be induced by cues associated to drug's rewarding effects.

CPP depends on the ability to learn and remember associations between drug and environment (Bardo and Bevins, 2000). Based on the findings of the present study and the effects of social interaction on cocaine CPP-reinstatement-associated Zif268 expression shown previously (Fritz et al., 2011a), it appears that social interaction regulates the conditioned effects of drugs (immediate early genes) rather than the long-lasting effects of drugs of abuse (ΔFosB). Thus, social interaction could weaken the ability of cocaine CPP-associated cues to trigger relapse.

Contrary to ΔFosB, previous work has shown that elevated CREB in the nucleus accumbens shell reduces cocaine- and morphine-induced conditioned place preferences (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002). Increased CREB function in the striatum has recently been implicated in reduced motivation to self-administer cocaine (Hollander et al., 2010). Furthermore, elevated CREB function in the nucleus accumbens shell produced increases in intracranial self-stimulation thresholds (Muschamp et al., 2011). However, although stress is known to increase the reinforcing effects of drugs and reinstate extinguished drug-seeking behavior in rats (Lu et al., 2003), stress (footshock) has been shown to activate CREB within the nucleus accumbens shell (Muschamp et al., 2011). In addition,

(Pliakas et al., 2001) showed that forced swim stress activates CREB via phosphorylation in the nucleus accumbens shell and that this neuroadaptive response has functional effects on behavior. Specifically, elevating CREB expression to mimic activation in the nucleus accumbens shell increased immobility behavior in the forced swim test whereas disruption of CREB in the nucleus accumbens shell produced opposite effects (Pliakas et al., 2001). Our results showed that social interaction during extinction of cocaine CPP increased pCREB expression in nucleus accumbens shell as compared to rats that undergo cocaine CPP in the absence of alternate social interaction during extinction. It is likely that CREB activation in the nucleus accumbens shell after social interaction in our study reflects a reduction of the rewarding effects of cocaine rather than stress-related effects. Indeed, while stress is known to induce reinstatement of extinguished drug-seeking behavior, we have shown that social interaction during extinction prevents reinstatement of cocaine CPP (Fritz et al., 2011a). Furthermore, it has been shown that social defeat or negative social interaction decreased functional activation as measured by zif268 mRNA expression in the medial prefrontal cortex (Covington et al., 2005) and that a temporary downregulation of zif268 expression in the medial prefrontal cortex of male rats produced social anxiety-like behaviors (Stack et al., 2010). In contrast, social interaction used in our study did not alter cocaine CPP-associated Zif268 expression in the medial prefrontal cortex (Fritz et al., 2011a). In addition, it has been shown that acute stress induced FosB/ΔFosB predominantly in the prefrontal cortex and the nucleus accumbens while chronic stress induced ΔFosB expression particularly in the prefrontal cortex, the nucleus accumbens and the basolateral amygdala (Perrotti et al., 2004). However, social interaction during extinction induced neither FosB/ΔFosB in any of the regions of the prefrontal cortex investigated nor ΔFosB expression in the prefrontal cortex or the basolateral amygdala. These evidences suggest that rats expressing cocaine CPP followed by social interaction during extinction did not exhibit signs of stress.

We expected to find a difference in pCREB expression after social interaction in the VTA. Unexpectedly, we found that social interaction during extinction increased pCREB in the cingulate cortex area 1 (Cg1). In support of this finding, it has been shown that morphine increased pCREB expression in the prefrontal cortex of alcohol-avoiding rats in comparison with alcohol non-avoiding rats (Kaste et al., 2009). To date, not much data has been generated on the role of pCREB in the cingulate cortex area. However, given the role of the cingulate cortex in decision-making (Schweimer and Hauber, 2006), our results suggest that the increased pCREB expression in Cg1 may be importantly involved in social interaction effects.

A few caveats for the interpretation of our study should be highlighted. First of all, in this study we focused on the correlation between the beneficial effects of social interaction and the changes in expression of transcription factors such as FosB and CREB. We did not investigate the functional effects of these changes on reinstatement of cocaine CPP. For example, Viral vector-mediated elevation or disruption of CREB expression within the nucleus accumbens shell could explore its eventual causal involvement in conditioned place preference to cocaine (Carlezon et al., 1998; Pliakas et al., 2001), forced swim stress (Pliakas et al., 2001), intracranial self-stimulation and fear conditioning (Muschamp et al., 2011). Thus, it would be important to study the causal relation between CREB and the protective effects of social interaction on reinstatement of cocaine CPP. In addition, the present study did not investigate the functional implication of discrete brain regions in the protective effects of social interaction on reinstatement of cocaine CPP. Interestingly, in a previous study, we showed that if rats were concurrently conditioned for place preference by pairing cocaine with one compartment and social interaction with the other (i.e., mutually exclusive stimulus presentation during training), pre-acquisition lesioning the AcbC or the basolateral amygdala shifted the animals' preference toward social interaction, whereas a bilateral shell lesion shifted the preference toward cocaine CPP (Fritz et al., 2011c). Given that both stimuli (15 min dyadic social interaction vs. 15 mg/kg i.p. cocaine) can produce equally strong CPP (Fritz et al., 2011a,c), these findings suggest a role of the nucleus accumbens shell in mediating alternative non-drug-(social interaction) associated conditioned contextual stimuli (Fritz et al., 2011c) that are in accordance with the present finding showing that social interaction during extinction increased the expression of pCREB in the shell of the nucleus accumbens. Also, in this study we focused on the naive group as the sole control group to interpret the reported neural correlates of the effects of social interaction on reinstatement of cocaine CPP. Therefore, other control groups that show different aspects of "conditioning" vs. "pharmacological" effects of cocaine before the reinstatement test should be added in future studies to better interpret the functional significance of the neural correlates of the "anti-relapse" effects of "friendly" social interaction.

Our results indicate that pCREB and FosB but not ΔFosB are implicated in the protective effect of social interaction against cocaine-induced reinstatement of CPP. Accordingly, it has been reported that gene expression after a short cocaine treatment, i.e., five injections (daily i.p. injections of cocaine 10 mg/kg for five days) is highly dependent on the actions of CREB and less dependent on ΔFosB (McClung and Nestler, 2003). However, long-term cocaine administration, i.e., 20 injections (cocaine 15 mg/kg for five days/week for four consecutive weeks) resulted in a gene expression profile that is highly dependent on ΔFosB and less dependent on CREB (McClung and Nestler, 2003). In conclusion, social interaction during extinction may (1) weaken the association between contextual cues and the rewarding effects of drugs of abuse and/or (2) reduce the drugs' rewarding effects. These findings suggest that social interaction, if offered in a context that is clearly distinct from the previously drug-associated one, may profoundly inhibit relapse to cocaine addiction.

ACKNOWLEDGMENTS

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Involvement of the insular cortex in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training

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Glucocorticoids are known to enhance the consolidation of memory of emotionally arousing experiences by acting upon a network of interconnected brain regions. Although animal studies typically do not consider the insular cortex (IC) to be part of this network, the present findings indicate that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. The specific glucocorticoid receptor (GR) agonist RU 28362 (3 or 10 ng in 0.5 μl) infused bilaterally into the IC of male Sprague–Dawley rats immediately after one-trial inhibitory avoidance training dose-dependently enhanced 48 h retention performance. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed by an increased number of cells expressing immunoreactivity for phosphorylated extracellular signal-regulated kinase 1/2 (pERK1/2). However, systemic administration of a memory-enhancing dose of corticosterone (1 mg/kg) after inhibitory avoidance training rapidly reduced the number of pERK1/2-positive cells in the IC, suggesting that glucocorticoid administration reduces overall neuronal activity of the IC. To investigate which components of the inhibitory avoidance training experience were influenced by the intra-IC glucocorticoid administration, in the last experiment rats were trained on a modified inhibitory avoidance task in which context exposure and footshock training occur on two sequential days. RU 28362 administration into the IC enhanced later retention when infused immediately after either the context or footshock training. Thus, these findings indicate that the IC mediates glucocorticoid effects on the consolidation of memory of different components of inhibitory avoidance training and suggest that the IC might be an important element of the rodent brain network involved in emotional regulation of learning and memory.

Keywords: stress, emotional memory, corticosterone, pERK, insula, glucocorticoid receptor

INTRODUCTION

Glucocorticoid hormones—stress hormones released from the adrenal cortex—are known to strengthen the consolidation of memory of emotionally arousing experiences (de Kloet et al., 1999; Roozendaal, 2000; Joëls et al., 2006; Sandi and Pinelo-Nava, 2007; de Quervain et al., 2009; Roozendaal and McGaugh, 2011; Schwabe et al., 2012). Most animal studies investigating glucocorticoid-induced enhancement of memory consolidation examined their effects on a network of interacting brain regions involved in emotional regulation of memory, including the basolateral amygdala (BLA), prefrontal cortex and hippocampus (Micheau et al., 1984; Roozendaal and McGaugh, 1997a,b, 2011; Roozendaal et al., 2008, 2009b; Miranda et al., 2008b). Although human neuroimaging studies generally support an involvement of these brain regions, as well as their functional interaction, in emotionally influenced learning and memory (Buchel et al., 1998; Richardson et al., 2004; Marschner et al., 2008; Shin and Liberzon, 2010), they also consistently point to a key role for the insular cortex (IC) (Craig, 2009; Menon and Uddin, 2010; Shin and Liberzon, 2010; Hartley et al., 2011; Ille et al., 2011). Extensive evidence indicates that the IC, which receives autonomic, visceral, and somatosensory inputs (Saper, 1982; Augustine, 1996), might be part of a "salience network" involved in the detection of novel and salient information (Downar et al., 2002; Seeley et al., 2007; Menon and Uddin, 2010) that is collectively upregulated in response to an acute stressful event and after highly stressful experiences (van Marle et al., 2010; Hermans et al., 2011; van Wingen et al., 2011). Accordingly, increased anterior insula activity has been reported during the subjective awareness of both positive and negative emotions (Craig, 2009; Menon and Uddin, 2010) as well as during the encoding and recall of a broad spectrum of emotionally salient learning tasks (Buchel et al., 1998; Alvarez et al., 2008, 2011; Marschner et al., 2008; King et al., 2009; Rasch et al., 2009).

Contrasting the human literature, the IC never received a prominent position in animal research investigating stress

hormone or emotional arousal effects on learning and memory. Although Bermudez-Rattoni and colleagues have reported several findings supporting the view that the IC might be involved herein (Bermudez-Rattoni et al., 1991, 1997, 2005; Bermudez-Rattoni and McGaugh, 1991; Nerad et al., 1996; Gutierrez et al., 1999; Miranda and Bermudez-Rattoni, 2007), most animal studies have been limited to investigating its involvement in the formation and maintenance of taste memory (Berman and Dudai, 2001; Bermudez-Rattoni et al., 2004; Shema et al., 2007; Nunez-Jaramillo et al., 2010; Stehberg et al., 2011). The present study investigated whether the IC is implicated in regulating glucocorticoid effects on the consolidation of memory of emotionally arousing inhibitory avoidance training. In the first experiment, we investigated whether the specific glucocorticoid receptor (GR) agonist RU 28362 administered into the IC immediately after one-trial inhibitory avoidance training enhances long-term retention of the training experience. Next, we examined whether a systemic injection of corticosterone, the major endogenous glucocorticoid in rodents, given immediately after inhibitory avoidance training recruits the IC and induces changes in cellular activity within this brain region. Cellular activity was assessed by determining phosphorylation levels of extracellular signal-regulated kinase 1/2 (pERK1/2), a signaling cascade implicated in neuronal activity and synaptic plasticity (Thomas and Huganir, 2004; Peng et al., 2010). To gain a better understanding of the precise role of the IC in regulating glucocorticoid effects on inhibitory avoidance memory, in the last experiment we employed a modified inhibitory avoidance procedure (Malin and McGaugh, 2006; Medina et al., 2007; Roozendaal et al., 2009b) that allows investigating which components of the inhibitory avoidance experience (i.e., memory of the context or the footshock) were influenced by the glucocorticoid infusion.

MATERIALS AND METHODS

SUBJECTS

Male adult Sprague–Dawley rats (280–320 g at time of surgery) from Charles River Breeding Laboratories (Kisslegg, Germany) were kept individually in a temperature-controlled (22°C) colony room and maintained on a standard 12 h light: 12 h dark cycle (07:00–19:00 h lights on) with *ad libitum* access to food and water. Training and testing were performed during the light phase of the cycle between 10:00 and 15:00 h. All procedures were in compliance with the European Community's Council Directive (86/609/EEC) and approved by the Institutional Animal Care and Use Committee of the University of Groningen, The Netherlands.

SURGERY FOR CANNULA IMPLANTATION

Animals, adapted to the vivarium for at least 1 week, were anesthetized with a subcutaneous injection of ketamine (37.5 mg/kg of body weight; Alfasan) and dexmedetomidine (0.25 mg/kg; Orion), and received the non-steroidal analgesic carprofen (4 mg/kg, s.c.; Pfizer). Oxygen (35%) mixed with ambient air was administered during surgery such that blood oxygenation levels would not drop below 90% (Fornari et al., 2012). The skull was positioned in a stereotaxic frame (Kopf Instruments), and two stainless-steel guide cannulae (15 mm; 23 gauge; Small Parts, Inc.) were implanted bilaterally with the cannula tips 2.0 mm above the anterior IC. The coordinates were based on the atlas of Paxinos and Watson (2007): anteroposterior: +1.0 mm from Bregma; mediolateral: ±5.5 mm from midline; dorsoventral: −4.8 mm from Bregma; incisor bar: −3.3 mm from interaural. The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (15 mm long 00 insect dissection pins), inserted into each cannula to maintain patency, were removed only for the infusion of drugs. After surgery, the rats received a subcutaneous injection of 3 ml of saline to facilitate clearance of drugs and prevent dehydration, and were subsequently administered atipamezole hydrochloride (Antisedan, 0.25 mg/kg, s.c.; Orion) to reverse anesthesia. The rats were allowed to recover for a minimum of 7 days before initiation of training and were handled three times for 1 min each during this recovery period to accustom them to the infusion procedure.

INHIBITORY AVOIDANCE APPARATUS AND PROCEDURES

For all experiments, rats were trained and tested in an inhibitory avoidance apparatus, consisting of a trough-shaped alley (91 cm long, 15 cm deep, 20 cm wide at the top, and 6.4 cm wide at the bottom) divided into two compartments, separated by a sliding door that opened by retracting into the floor (McGaugh et al., 1988). The starting compartment (30 cm) was made of opaque white plastic and was well lit; the shock compartment (60 cm) was made of dark, electrifiable metal plates and was not illuminated. Training and testing were conducted in a sound- and light-attenuated room.

For one-trial inhibitory avoidance training, the rats were placed in the starting compartment of the apparatus, facing away from the door, and were allowed to enter the dark (shock) compartment. After the rat stepped completely into the dark compartment, the sliding door was closed and a single inescapable footshock (0.35 mA; 1 s) was delivered. The rats were removed from the shock compartment 15 s later and, after drug treatment, returned to their home cages. For the modified, two-phase inhibitory avoidance procedure (Malin and McGaugh, 2006; Roozendaal et al., 2009b), on the first day (context training), the rat was placed into the starting compartment, facing away from the door, and allowed to freely explore the inhibitory avoidance apparatus for 3 min. On day 2 (shock training), each rat was placed directly into the dark compartment, facing away from the starting compartment, with the retractable door closed. The rat then received an inescapable footshock (0.75 mA; 1 s) and immediately afterward was removed from the training apparatus. For both one-trial and two-phase inhibitory avoidance, retention was tested 48 h after training by placing the rat into the starting compartment of the inhibitory avoidance apparatus and measuring the latency to re-enter the former shock compartment with all four paws (maximum latency of 600 s). Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

LOCAL DRUG INFUSION INTO THE INSULAR CORTEX

The specific GR agonist RU 28362 (11β,17β-dihydroxy-6,21-dimethyl-17α-pregna-4,6-trien-20yn-3-one, 3 or 10 ng; a generous gift of Aventis, Frankfurt, Germany) was first dissolved in 100% ethanol and subsequently diluted in 0.9% saline to reach

a final ethanol concentration of 0.5%. Receptor binding studies have shown that this compound has selective and high affinity for GRs (Teutsch et al., 1981). Bilateral infusions of RU 28362 or an equivalent volume of vehicle (0.5% ethanol in saline) into the IC were given immediately after one-trial inhibitory avoidance training or after either the context or footshock components of the modified, two-phase inhibitory avoidance task by using 30 gauge injection needles connected to a 10 μl Hamilton microsyringe with polyethylene (PE-20) tubing. The injection needle protruded 2.0 mm beyond the tip of the cannula and a 0.5 μl injection volume was infused over a period of 50 s by an automated syringe pump (Stoelting Co). The injection needles were retained within the cannulae for an additional 20 s after drug infusion to maximize diffusion and to prevent backflow of drug into the cannulae. The infusion volume was based on previous findings indicating that infusion of this volume into the IC (Miranda et al., 2008a; Roozendaal et al., 2010), but not the cortex dorsal to the IC (Bermudez-Rattoni et al., 2005), modulates memory consolidation. To control for time- and site-specificity, additional groups of rats received delayed infusions of RU 28362 or vehicle into the IC 3 h after the training trial or immediate post-training infusions into the somatosensory cortex, located approximately 1 mm dorsal to the IC. The use of post-training drug administration provides direct support for the view that the treatment affects memory consolidation processes and that retention performance is, thus, not confounded by possible effects on attentional, motivational, or sensory-perceptual mechanisms at the time of training or test (McGaugh, 1966).

SYSTEMIC CORTICOSTERONE TREATMENT

Corticosterone (1 mg/kg, Sigma-Aldrich) or vehicle, in a volume of 2 ml/kg body weight, was given subcutaneously immediately after the training trial. Corticosterone was dissolved in 5% ethanol in saline. This dose of corticosterone is known to enhance memory consolidation of different types of training (Hui et al., 2004; Okuda et al., 2004; Miranda et al., 2008a).

CANNULA PLACEMENT VERIFICATION

Rats were deeply anesthetized with an overdose of sodium pentobarbital (≈100 mg/kg, i.p.) and perfused transcardially with a 0.9% saline solution followed by 4% formaldehyde. Following decapitation, the brains were removed and immersed in fresh 4% formaldehyde. At least 24 h before sectioning, the brains were submerged in a 25% sucrose (wt/vol) solution in water for cryoprotection. Coronal sections of 50 μm were cut on a cryostat, mounted on gelatin-coated slides, and stained with cresyl violet. The sections were examined under a light microscope and determination of the location of injection needle tips in the IC was made according to the atlas plates of Paxinos and Watson (2007), by an observer blind to drug treatment condition. Rats with injection needle placements outside the IC or with extensive tissue damage at the injection needle tips were excluded from analysis.

IMMUNOHISTOCHEMISTRY

Thirty minutes after training and systemic corticosterone treatment, rats were perfused transcardially with ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.4, followed by ice-cold 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4. The brains were removed, post-fixed overnight at 4°C, and then transferred to a 25% sucrose solution in 0.1 M PB for 3–6 days at 4°C. Frozen coronal sections at the level of the anterior IC (0.2–2.5 mm anterior to Bregma) were cut at a thickness of 20 μm on a cryostat and collected in Tris-buffered saline (TBS) with 0.1% sodium azide and phosphatase inhibitors (20 mM sodium fluoride and 2 mM sodium orthovanadate).

Every eighth section was used for quantification. Free-floating sections were rinsed in 0.3% Triton X-100 in TBS with phosphatase inhibitors. To block non-specific binding, sections were incubated with TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 5% normal donkey serum (nds, Jackson ImmunoResearch) for 1 h. Subsequently, sections were incubated with a cocktail of primary antibodies for pERK1/2 (rabbit anti-phospho-p44/42 MAP kinase, 1:1000; cell signaling #9101) and NeuN (mouse anti-NeuN, 1:100; Millipore Mab 377) or pERK1/2 and CaMK II (mouse anti-calcium/calmodulin-dependent protein kinase II, 1:400; Millipore #05–532) in TBS containing phosphatase inhibitors, 0.3% Triton X-100 and 1% nds for 48 h at 4°C. Sections were then rinsed several times with TBS and incubated with a cocktail of specific fluorochrome-conjugated antibodies (Alexa Fluor 488 donkey anti-rabbit 1:500 and Alexa Fluor 594 donkey anti-mouse 1:1000 or Alexa Fluor 633 goat anti-rabbit 1:500 and Alexa 488 donkey anti-mouse 1:500; Invitrogen) for 2 h in the dark. Sections were rinsed again in TBS, incubated with Hoechst dye (0.1 μg/ml, Invitrogen) for 30 min and mounted on gelatin-coated slides, using non-fading mounting medium for coverslipping and stored in the dark for further analysis.

Images of each section were acquired with TissueFaxs®, Zeiss AxioObserver Z1 Microscope System (Tissue-Gnostics GmbH, Vienna, Austria). The number of pERK1/2 immunopositive nuclei was quantified with ImageJ 1.43 m software (Girish and Vijayalakshmi, 2004; Papadopulos et al., 2007). Cell counts were determined at two levels within the anterior IC (2.5–1.7 mm and 1.6–0.7 mm anterior to Bregma), according to the standard atlas plates of Paxinos and Watson (2007), and expressed as number of pERK1/2-positive nuclei per mm^2. Quantitative analysis of cell counts was performed blind to treatment condition. Double-labeling was examined with a Leica SP2 AOBS confocal microscope.

STATISTICS

Data are expressed as mean ± SEM. Inhibitory avoidance training and retention test latencies were analyzed with One-Way ANOVAs. Further analysis used Fisher's *post-hoc* tests to determine the source of the detected significances. To determine whether learning had occurred, paired *t*-tests were used to compare the training and retention latencies. Quantitative measures of pERK1/2 immunoreactivity in the IC were analyzed with Two-Way-ANOVAs, using Training (training vs. no training) and Corticosterone treatment (vehicle vs. corticosterone) as independent factors. The analyses were followed by Fisher's *post-hoc* tests, when appropriate. Individual comparisons with home-cage control groups were performed with Student *t*-tests for independent samples. For all comparisons, a probability level of < 0.05 was

accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

GR AGONIST ADMINISTRATION INTO THE INSULAR CORTEX ENHANCES MEMORY CONSOLIDATION OF INHIBITORY AVOIDANCE TRAINING

This experiment examined whether the GR agonist RU 28362 infused into the IC enhances the consolidation of memory of inhibitory avoidance training. For that, bilateral infusions of RU 28362 (3 or 10 ng in 0.5 μl) or vehicle were administered into the IC immediately after one-trial inhibitory avoidance training and retention of the training was tested 48 h later. Control groups received delayed infusions of the GR agonist into the IC 3 h after the training trial to determine whether the GR agonist enhances retention by influencing time-dependent processes underlying memory consolidation.

Average step-through latencies for all groups during training, before footshock or drug treatment, were 9.9 ± 0.8 s (mean ± SEM). One-Way ANOVA for training latencies revealed no significant differences between groups [$F(2, 31) = 0.17$, $p = 0.84$, data not shown]. Forty-eight hour retention latencies of rats administered vehicle into the IC immediately after training were significantly longer than their latencies during the training trial (paired t-test: $p < 0.05$), indicating that the rats retained memory of the inhibitory avoidance experience. As is shown in **Figure 1A**, rats treated with the GR agonist immediately after training had significantly longer retention test latencies as compared with rats that received vehicle [$F(2, 31) = 3.59, p < 0.05$]. Fisher's *post-hoc* tests revealed that the lower dose of RU 28362 (3 ng) enhanced retention ($p < 0.05$, compared with vehicle), whereas retention latencies of animals given the higher dose (10 ng) approached, but failed to reach, significance ($p = 0.07$). As is shown in **Figure 1B**, the GR agonist administered into the IC 3 h after the training did not significantly alter retention latencies [$F(2, 16) = 0.26$, $p = 0.78$], indicating a time-limited involvement of the IC in mediating glucocorticoid effects on the consolidation of inhibitory avoidance memory.

Figures 1C,D show cannula placement within the IC. All injection needle tips of rats included in the analysis were localized within the granular and dysgranular subdivisions of the IC. To control for site specificity, other groups of rats received immediate post-training infusions of vehicle or the GR agonist (3 or 10 ng in 0.5 μl) into the somatosensory cortex, approximately 1 mm above the IC (see, **Figure 1C**). One-Way ANOVA for 48 h retention latencies revealed no significant GR agonist effect [$F(2, 12) = 0.29$, $p = 0.75$, **Figure 1E**], indicating that the memory-modulatory effects of RU 28362 are localized within the IC.

SYSTEMIC CORTICOSTERONE ADMINISTRATION IMMEDIATELY AFTER ONE-TRIAL INHIBITORY AVOIDANCE TRAINING REDUCES pERK1/2-IMMUNOREACTIVITY IN THE INSULAR CORTEX

The findings described above indicate that direct pharmacological activation of GRs in the IC after inhibitory avoidance training enhances the consolidation of memory of this experience in a dose-, time- and site-specific manner. However, these findings do not indicate whether circulating glucocorticoids normally act upon the IC in regulating memory consolidation. Therefore, the next experiment examined whether a systemic injection of corticosterone after inhibitory avoidance training changes neuronal activity of the IC. Rats received a subcutaneous injection of corticosterone (1 mg/kg) or vehicle immediately after one-trial inhibitory avoidance training (or without training) and were sacrificed 30 min later. Brains were processed to investigate training- and drug-induced changes in the number of pERK1/2-positive cells at two locations within the anterior IC. ERK1/2, a member of the mitogen-activated protein (MAP) kinase family, is considered to be phosphorylated by elevated neuronal activity and synaptic plasticity (Thomas and Huganir, 2004; Peng et al., 2010). Home-cage control groups did not receive any training or drug treatment. Other rats received the same post-training injection of corticosterone or vehicle and their retention was tested 48 h later.

As is shown in **Figure 2A**, post-training injection of this dose of corticosterone significantly enhanced 48 h retention latencies ($p < 0.05$). **Figure 2B** shows the pattern of pERK1/2 expression in the IC. Immunoreactivity for pERK1/2 was found in somata as well as fibers (**Figure 2C**). Qualitative double-labeling indicated that the vast majority of pERK1/2-positive cells in the IC also showed immunoreactivity for the neuronal marker NeuN (**Figure 2C**) or CaMKII (**Figure 2D**), a marker for glutamatergic pyramidal cells (McDonald et al., 2002). These observations strongly suggest that pERK1/2 immunoreactivity within somata was mainly expressed in glutamate-rich pyramidal cells. Because most pERK1/2 immunoreactivity was concentrated in the superficial layers (II–III) of the agranular and dysgranular IC, cell counts were restricted to these regions, at two levels (2.5–1.7 mm and 1.6–0.7 mm anterior to Bregma), according to the standard atlas plates of Paxinos and Watson (2007). **Figure 2E** shows the number of pERK1/2-positive cells at these two levels of the IC of rats treated with corticosterone or vehicle immediately after inhibitory avoidance training or of rats that were not trained. Across all groups, the number of pERK1/2-positive cells at the more rostral level appeared to be higher than that at the more caudal level. At the rostral level, Two-Way ANOVA indicated a significant training effect [$F(1, 14) = 11.63, p < 0.01$] but no corticosterone effect [$F(1, 14) = 1.95$, $p = 0.18$] or interaction effect between these two parameters [$F(1, 14) = 0.53$, $p = 0.48$]. Fisher's *post-hoc* analyses indicated that rats that were trained on the inhibitory avoidance task and subsequently treated with vehicle had more pERK1/2-positive cells than non-trained vehicle-treated rats ($p < 0.05$). Corticosterone administration after inhibitory avoidance training did not significantly alter the number of pERK1/2-positive cells as compared to vehicle-treated rats. When compared to home-cage controls, student t-test analyses indicated that rats treated with either vehicle ($p < 0.05$) or corticosterone ($p < 0.05$) immediately after training had more pERK1/2-positive cells, whereas the number of pERK1/2-positive cells of non-trained rats administered vehicle or corticosterone did not differ from those of home-cage controls.

On the other hand, Two-Way ANOVA for the number of pERK1/2-positive cells at the more caudal level revealed significant effects of training [$F(1, 14) = 21.66$, $p < 0.01$], corticosterone treatment [$F(1, 14) = 10.55$, $p < 0.01$] and the

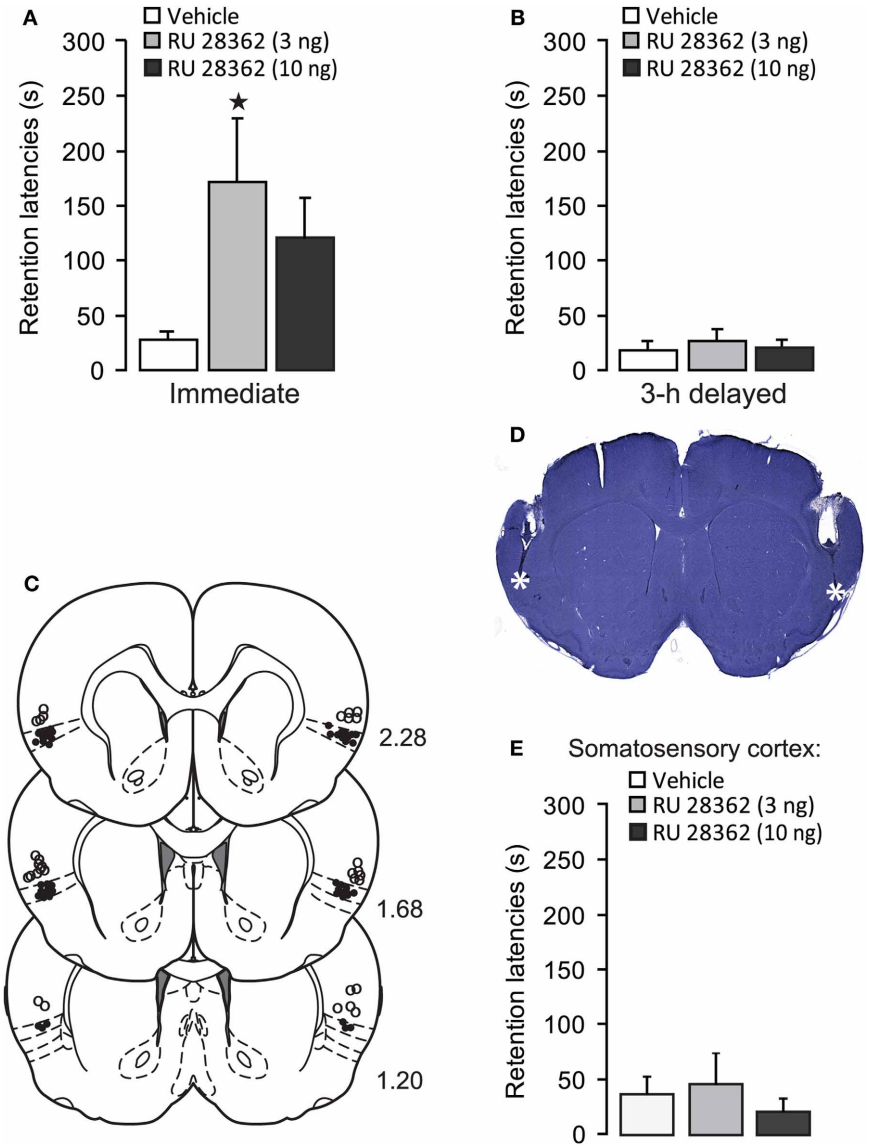

FIGURE 1 | Glucocorticoid receptor agonist administration into the insular cortex enhances memory consolidation of inhibitory avoidance training. (A) Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 μl) into the IC immediately after training. $*p < 0.05$ as compared with the vehicle-treated group ($n = 11$–12 per group). **(B)** Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 μl) into the IC 3 h after training ($n = 6$–7 per group). **(C)** Location of injection needle tips within the IC of all rats included in the immediate infusions groups (black circles) and 15 rats with infusion needle tips in the somatosensory cortex as a control for site specificity (open circles). Adapted from Paxinos and Watson (2007). **(D)** Representative photomicrograph illustrating placement of cannulae and needle tips within the insular cortex. **(E)** Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 μl) into the somatosensory cortex, approximately 1 mm above the IC, immediately after training ($n = 4$–6 per group).

interaction between them [$F(1, 14) = 6.64, p < 0.05$, **Figure 2E**]. Fisher's *post-hoc* analyses indicated that trained rats that were subsequently injected with vehicle had significantly more pERK1/2-positive cells than rats that were administered corticosterone after training ($p < 0.01$) or non-trained rats treated with either vehicle or corticosterone ($p < 0.01$). When compared to home-cage controls, the group that received vehicle immediately after training was the only one that showed increased cell counts ($p < 0.0001$). These findings indicate that training on the inhibitory avoidance task increases the number of pERK1/2-positive cells within this area of the IC, and that post-training administration of a memory-enhancing dose of corticosterone resulted in a significant decrease in the number of pERK1/2-positive cells (as compared to vehicle-treated trained rats), which did not differ significantly from home-cage controls or non-trained rats.

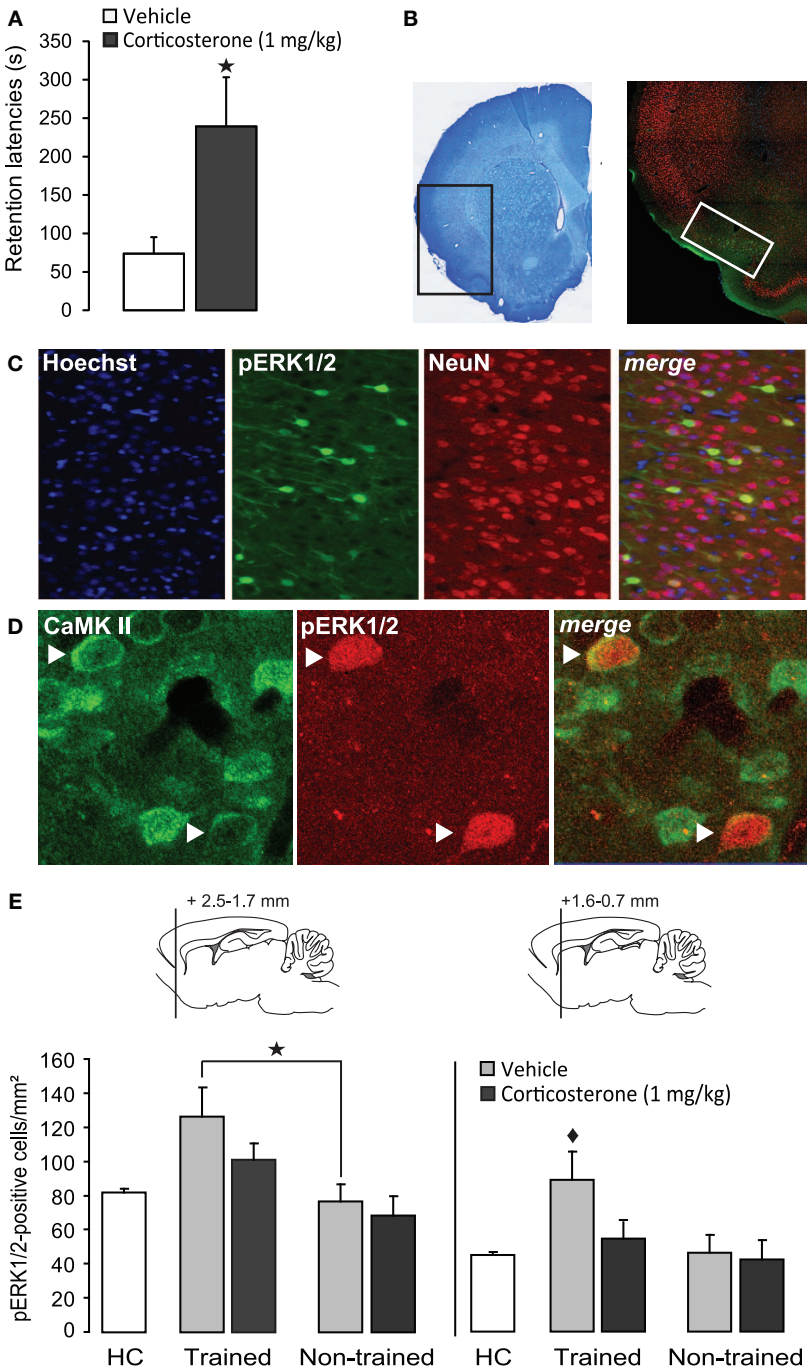

FIGURE 2 | Systemic injection of a memory-enhancing dose of corticosterone after one-trial inhibitory avoidance training reduces pERK1/2-immunoreactivity in the insular cortex. (A) Step-through latencies (mean ± SEM) in seconds on a 48 h inhibitory avoidance retention test of rats given a subcutaneous injection of corticosterone (1 mg/kg) or vehicle immediately after training. $^{*}p < 0.05$ ($n = 8$–9 per group). **(B)** Example of a Nissl-stained coronal section depicting the region that was acquired for the analyses of immunostaining and the distribution pattern of pERK1/2 expression in the insular cortex. The white square indicates the region analyzed for cell counts. **(C)** Triple localization of Hoechst (blue), pERK1/2 (green) and the neuronal marker NeuN (red) in the insular cortex. Merging the three channels demonstrates that all pERK1/2-positive cells exhibit immunoreactivity for NeuN. **(D)** Dual localization of pERK1/2 (red) and CamKII (green) immunoreactivity in the insular cortex. Merging of the red and green channels demonstrates that pERK1/2-positive cells also showed immunoreactivity for CamKII. Arrows point to cells with combined pERK1/2 and CamKII immunoreactivity. **(E)** Number of pERK1/2-positive cells (mean ± SEM) at two levels within the insular cortex as assessed 30 min after inhibitory avoidance training and systemic injection of corticosterone ($n = 4$) or vehicle ($n = 4$). Non-trained rats received an injection of corticosterone ($n = 5$) or vehicle ($n = 5$) but were not trained on the inhibitory avoidance task. Home-cage (HC) control rats ($n = 6$) did not receive any training or systemic injection. $^{*}p < 0.05$ as compared with non-trained vehicle-treated rats. $\blacklozenge p < 0.01$ as compared with all other groups.

GR AGONIST INFUSIONS INTO THE INSULAR CORTEX ENHANCE MEMORY FOR BOTH THE CONTEXT AND FOOTSHOCK COMPONENTS OF INHIBITORY AVOIDANCE TRAINING

The findings described above indicate that the IC is an important target structure for glucocorticoids in regulating memory consolidation of inhibitory avoidance training. However, with one-trial inhibitory avoidance it is not possible to investigate the relative involvement of a brain region in memory consolidation of the contextual information independently from that of the footshock. To address this issue, a modified two-phase inhibitory avoidance training procedure had been developed in which context training and footshock training occur on two sequential days (Malin and McGaugh, 2006; Medina et al., 2007; Roozendaal et al., 2009b). In the next experiment, we used this modified inhibitory avoidance training procedure to investigate whether GR agonist administration into the IC enhances memory consolidation of contextual information, the footshock experience, or of both components of training.

Figure 3 shows 48 h retention latencies of rats administered the GR agonist (3 or 10 ng in 0.5 μl) into the IC after either the context or footshock component of inhibitory avoidance training. One-Way ANOVAs for retention latencies revealed significant group effects of rats given intra-IC infusions of RU 28362 immediately after either context [$F(2, 37) = 3.50$, $p < 0.05$] or footshock training [$F(2, 37) = 4.04$, $p < 0.05$]. Retention latencies of rats given intra-IC infusions of the 3 and 10 ng doses of RU 28362 immediately after context training (both, $p < 0.05$), or infusions of the 3 ng dose of RU 28362 after footshock training ($p < 0.01$) were significantly longer than those of their respective vehicle controls. Importantly, as is shown in **Table 1**, both doses of RU 28362 administered into the IC after either context or shock training to animals that did not receive the other component of training did not enhance 48 h retention latencies [context: $F(2, 15) = 0.70$, $p = 0.51$; footshock: $F(2, 15) = 0.88$, $p = 0.44$], indicating that the expression of the enhanced memory depends on the learning about both context and footshock.

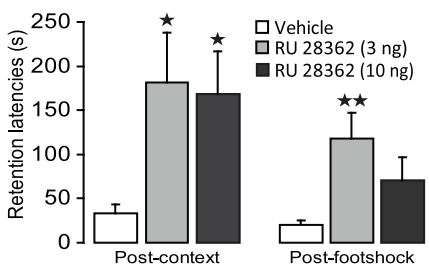

FIGURE 3 | Glucocorticoid receptor agonist infusions into the insular cortex enhance memory consolidation of both the context and footshock components of inhibitory avoidance training. Step-through latencies (mean ± SEM) in seconds on the 48 h inhibitory avoidance retention test of rats given bilateral infusions of the glucocorticoid receptor agonist RU 28362 (3 or 10 ng in 0.5 μl) into the insular cortex immediately after either context or shock training on the two-phase modified inhibitory avoidance task ($n = 12$–14 per group). $^{*}p < 0.05$, $^{**}p < 0.01$, as compared with the corresponding vehicle-treated group.

Table 1 | Effect of RU 28362 after context or footshock exposure alone.

	Vehicle	RU 28362 (3 ng)	RU 28362 (10 ng)	*p*
Context exposure alone	4.2 ± 0.9 (5)	3.1 ± 0.3 (7)	3.2 ± 0.9 (6)	0.51
Footshock exposure alone	8.7 ± 4.2 (7)	19.0 ± 5.2 (6)	13.0 ± 8.3 (5)	0.44

Data are presented as mean ± SEM. Number of animals per group is shown in parentheses.

DISCUSSION

The aim of this study was to investigate whether the IC is involved in regulating glucocorticoid effects on memory consolidation of inhibitory avoidance training. Our findings indicate that the specific GR agonist RU 28362 infused bilaterally into the IC after inhibitory avoidance training induced dose- and time-dependent enhancement of the consolidation of memory of this training experience. Moreover, training on the inhibitory avoidance task increased neuronal activity of the IC, as assessed with immunoreactivity for pERK1/2. Unexpectedly, systemic injection of a memory-enhancing dose of corticosterone after inhibitory avoidance training rapidly reduced pERK1/2 expression in the IC. Lastly, we show that post-training activation of GRs in the IC enhanced the consolidation of memory of both the footshock and contextual components of inhibitory avoidance training. These findings provide evidence that the IC is an important target structure for glucocorticoids in regulating the consolidation of different components of inhibitory avoidance memory.

It is well established that glucocorticoid hormones, via GR activation, enhance memory consolidation of emotionally arousing training experiences (de Kloet et al., 1999; Sandi and Pinelo-Nava, 2007; Roozendaal et al., 2009a; Schwabe et al., 2012). However, most studies investigating glucocorticoid effects on the formation and stabilization of memory, and its molecular underpinnings, examined glucocorticoid actions on a network of interconnected brain regions such as the BLA and hippocampus (Revest et al., 2005; Sandi and Pinelo-Nava, 2007; Roozendaal et al., 2009b; Groeneweg et al., 2011). Surprisingly little is known concerning glucocorticoid effects on the IC or other cortical regions in regulating learning and memory. Our finding that RU 28362 infused into the IC immediately after inhibitory avoidance training enhanced later retention is in line with other recent evidence indicating that corticosterone or GR agonist administration into the IC also enhances the consolidation of memory of conditioned taste aversion (Miranda et al., 2008a) and object recognition training (Roozendaal et al., 2010). More generally, findings from human and, to a lesser extent, animal studies indicate that the IC is involved in memory formation of emotionally arousing experiences. Human neuroimaging studies reported activation of the IC during the encoding of aversive conditioning paradigms (Buchel et al., 1998; Alvarez et al., 2008, 2011; Marschner et al., 2008). In rats, lesions of the IC, made before training, disrupted memory of emotionally arousing water-maze spatial (Bermudez-Rattoni et al., 1991; Nerad et al., 1996) and inhibitory avoidance training (Bermudez-Rattoni and McGaugh, 1991). Although such

neuroimaging studies in humans and permanent lesion studies in animals obviously cannot determine whether the memory effects were attributable to specific influences on memory consolidation, our findings using immediate post-training or delayed drug delivery provide compelling evidence for the view that the IC is involved in time-dependent processes underlying the consolidation of memory (Bermudez-Rattoni et al., 1991, 2005; Gutierrez et al., 1999; Miranda and McGaugh, 2004; Roozendaal et al., 2010).

To investigate whether inhibitory avoidance training normally recruits the IC, we examined the pattern of pERK1/2 expression in this cortical region 30 min after inhibitory avoidance training. ERK is a rapidly activated protein that has been implicated in neuronal activity as well as neuroplasticity and memory consolidation (Atkins et al., 1998; Schafe et al., 2000; Thomas and Huganir, 2004). Exposure to the inhibitory avoidance training procedure increased the number of pERK1/2-positive cells within the IC of rats treated with vehicle. Qualitative immuno-staining indicated that most pERK1/2-positive cells were glutamate-rich pyramidal cells. These findings are consistent with the evidence that ERK1/2 is activated in neurons in response to excitatory glutamatergic activity (Fiore et al., 1993; Xia et al., 1996), and suggest that the elevated pERK1/2 immunoreactivity reflects an increased neuronal activity within this region (Fujita et al., 2010). Such findings are in agreement with the above-mentioned human neuroimaging studies indicating enhanced IC activity during affective processing and the encoding of emotionally arousing information (Buchel et al., 1998; Alvarez et al., 2008, 2011; Marschner et al., 2008; King et al., 2009; Rasch et al., 2009; Hermans et al., 2011). The pattern of pERK1/2 expression in the present study was similar to that of Kobayashi et al. (2010) reporting that novel sucrose stimulation increased the number of pERK1/2-positive cells in the IC, when compared to sucrose-experienced rats. As in our study, they found that pyramidal neurons, but not GABAergic interneurons, in the upper layers of the IC were most susceptible to ERK1/2 phosphorylation. However, the difference between naïve and sucrose-experienced rats was only found in the gustatory region—dysgranular and granular subdivisions—of the IC (Kobayashi et al., 2010), whereas in the present study most pERK1/2 immunoreactivity was concentrated in the agranular and dysgranular regions, and not in the gustatory region. Other studies also indicated increased pERK1/2 levels in the IC in response to an unfamiliar taste stimulus (Berman et al., 1998, 2000), and that such activation is necessary for taste learning (Berman et al., 1998). These findings suggest that ERK1/2 activation in the IC may contribute to the detection of novelty and/or memory formation (Berman et al., 1998; Bermudez-Rattoni, 2004; Kobayashi et al., 2010).

A more puzzling observation is that a memory-enhancing dose of corticosterone administered immediately after inhibitory avoidance training resulted in a rapid decrease in the number of pERK1/2-positive cells within the IC. Corticosterone administration to non-trained rats did not induce such a reduction. The selective influence of corticosterone on trained, and not naïve, rats is consistent with prior evidence indicating that RU 28362 infused into the IC interacts with training-induced noradrenergic activation in enhancing memory consolidation (Roozendaal et al., 2010). However, the direction of the corticosterone effect on pERK1/2 immunoreactivity appears to contrast prior evidence indicating that glucocorticoid effects on memory consolidation involving other brain regions require an upregulation of pERK1/2-MAP kinase activity. Previously, we reported that a memory-enhancing dose of RU 28362 infused into either the BLA or medial prefrontal cortex after inhibitory avoidance training rapidly elevated pERK1/2 levels in the other brain site (medial prefrontal cortex or BLA, respectively) (Roozendaal et al., 2009b). Moreover, stress and corticosterone are known to interact with training-associated glutamatergic activity within the hippocampus to increase the expression and enzymatic activity of the MAP kinase pathway (Revest et al., 2005; Gutierrez-Mecinas et al., 2011). A blockade of pERK1/2 signaling with a MEK inhibitor infused into any of these brain regions prevented glucocorticoid effects on memory consolidation. Thus, although we only examined pERK1/2 immunoreactivity in somata, and not in fibers, these findings suggest that glucocorticoids, at least at a 30 min time interval, might exert an opposite influence on the pERK1/2-MAP-kinase pathway, and possibly neuronal activity, in the IC as compared to the BLA or hippocampus. Emerging evidence supports the view that glucocorticoids can activate or suppress synaptic plasticity and neuronal activity in a highly time-dependent and brain region-specific manner (Joëls et al., 2011).

Our finding that corticosterone-induced enhancement of inhibitory avoidance memory is associated with a reduced pERK1/2 expression, and possibly neuronal activity, of the IC is in agreement with that of a recent functional magnetic resonance imaging study in humans showing that the combined oral administration of cortisol and the noradrenergic stimulant yohimbine shortly before the encoding of emotionally arousing pictures led to a strong deactivation of the IC, along with the hippocampus and orbitofrontal cortex (van Stegeren et al., 2010). Moreover, the magnitude of this deactivation correlated with enhanced recall of the material assessed 1 week later. Highly comparable, another study reported that human participants who responded with a large increase in cortisol when confronted with a psychosocial stressor, as opposed to low-responders, also showed deactivation of a network of limbic regions that includes the right anterior insula (Pruessner et al., 2008). The crucial question is whether and how such overall deactivation of the IC might contribute to the enhanced consolidation of memory processing. Pruessner et al. (2008) interpreted the deactivation of limbic regions in their study as a stress-mediated regulatory influence on hypothalamic-pituitary-adrenal (HPA) axis activity. However, no findings are available indicating whether or not the IC per se is involved in HPA-axis regulation. Alternatively, based on the involvement of frontal areas, including the anterior IC, in emotional regulation, attention, and focusing (Dolcos et al., 2004, 2007), van Stegeren et al. (2010) suggested that a reduced BOLD signal in these areas could reflect either a loss of top-down inhibition, and therefore activation (disinhibition) of other brain regions, or an increased signal-to-noise ratio, resulting in a shift of attentional processing from a peripherally to a centrally focused view. In line with this hypothesis, others have proposed that the anterior IC is part of a "salience network" which detects salient stimuli (events) and triggers appropriate control signals to regulate

behavior and homeostatic state (Downar et al., 2002; Seeley et al., 2007; Menon and Uddin, 2010). It is possible, therefore, that a reduced neuronal activity of the IC after exogenous glucocorticoid administration induces a "reallocation of resources," thereby increasing the detection of the most relevant stimuli and enhancing the consolidation of memory of these experiences (Cahill and van Stegeren, 2003; van Stegeren et al., 2010).

Such a potential role of the IC, as part of the salience network, in novelty and salience detection fits well with our finding that local activation of GRs with RU 28362 enhanced the memory of exposure to both the contextual and aversive components of inhibitory avoidance training. Similarly, findings of human neuroimaging studies indicated activation of the IC in fear conditioning studies during the presentation of either specific conditioned stimuli or context (Buchel et al., 1998, 1999; Alvarez et al., 2008; Marschner et al., 2008). A broad involvement of the IC in regulating the consolidation of memory of emotionally salient experiences fits also well with other findings of animal studies indicating that post-training pharmacological manipulation of IC activity modulates memory consolidation of training on many different kinds of learning tasks, including spatial water-maze (Gutierrez et al., 1999), conditioned taste aversion (Miranda and McGaugh, 2004; Miranda et al., 2008a,b; Shema et al., 2009) and object recognition (Bermudez-Rattoni et al., 2005; Roozendaal et al., 2010). The extensive network of connections between the IC and other cortical regions, including the prefrontal, cingulate, perirhinal and entorhinal cortices (Mufson et al., 1981; Augustine, 1996; Shi and Cassell, 1998b; Hoistad and Barbas, 2008), might account for such a general modulatory influence on memory. There are also dense reciprocal connections between the IC and BLA (McDonald and Jackson, 1987; Shi and Cassell, 1998a,b). Extensive evidence indicates that the BLA influences memory consolidation of emotionally arousing training experiences by regulating neuroplasticity and information storage processes in other brain regions, including the hippocampus and dorsal striatum (McGaugh, 2002; Roozendaal and McGaugh, 2011). Therefore, it is possible that the IC and BLA share a functional commonality and cooperate in regulating memory consolidation. Indeed, some findings provide evidence for a necessary interaction between the IC and BLA in strengthening the consolidation of memory of emotionally arousing training. Post-training infusion of a cAMP analog into the IC is known to enhance memory consolidation of both conditioned taste aversion and inhibitory avoidance training. However, concurrent blockade of noradrenergic transmission in the BLA with the β-adrenoceptor antagonist propranolol prevents this memory enhancement (Miranda and McGaugh, 2004). Findings of human studies indicating an increased connectivity between the amygdala and IC during the encoding of emotionally arousing material (Rasch et al., 2009) support a functional interaction between the BLA and IC in regulating memory consolidation.

In summary, the present findings provide evidence for the view that the IC is importantly involved in regulating glucocorticoid effects on memory consolidation of emotionally arousing inhibitory avoidance training. These findings indicate that the IC deserves a prominent position in animal research investigating the neural basis of emotional regulation of learning and memory.

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Extinction procedure induces pruning of dendritic spines in CA1 hippocampal field depending on strength of training in rats

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Numerous reports indicate that learning and memory of conditioned responses are accompanied by genesis of dendritic spines in the hippocampus, although there is a conspicuous lack of information regarding spine modifications after behavioral extinction. There is ample evidence that treatments that typically produce amnesia become innocuous when animals are submitted to a procedure of enhanced training. We now report that extinction of inhibitory avoidance (IA), trained with relatively low foot-shock intensities, induces pruning of dendritic spines along the length of the apical dendrites of hippocampal CA1 neurons. When animals are trained with a relatively high foot-shock there is a high resistance to extinction, and pruning in the proximal and medial segments of the apical dendrite are seen, while spine count in the distal dendrite remains normal. These results indicate that pruning is involved in behavioral extinction, while maintenance of spines is a probable mechanism that mediates the protecting effect against amnesic treatments produced by enhanced training.

Keywords: hippocampus, dendritic spines, pruning, extinction, inhibitory avoidance, post-traumatic stress disorder

INTRODUCTION

It has been reported that treatments that typically produce amnesia become innocuous when administered to animals that have been submitted to an enhanced learning experience. Thus, generalized interference with cholinergic or serotonergic central activity produces amnesia of inhibitory avoidance (IA) trained with relatively low levels of foot-shock, but memory remains intact when higher levels of stimulation are used during training (e.g., Duran-Arevalo et al., 1990; Galindo et al., 2008). Similarly, long-term memory deficits of IA are produced by hindrance of synaptic activity of the striatum, amygdala, and substantia nigra; in contrast, under this circumstance memory is spared after enhanced IA training (Giordano and Prado-Alcalá, 1986; Pérez-Ruíz and Prado-Alcalá, 1989; Parent et al., 1992, 1995; Parent and McGaugh, 1994; Prado-Alcalá, 1995; Cobos-Zapiaín et al., 1996; Salado-Castillo et al., 2011). Recently, it was found that enhanced IA training prevented the typical amnesic effects of cycloheximide, a protein synthesis inhibitor (Díaz-Trujillo et al., 2009). In spite of this evidence, the neurobiological mechanisms of the protective effect of enhanced learning against amnesic treatments remain completely unknown.

The hippocampus is also involved in memory consolidation of IA training, as shown by the deleterious effects of interference with hippocampal activity on retention of this task (Izquierdo et al., 1992; Ambrogi-Lorenzini et al., 1996; Stubley-Weatherly et al., 1996; Martínez et al., 2002), although enhanced learning of this task also protects against amnesia produced by reversible blockade of hippocampal activity (Quiroz et al., 2003).

One structural neuronal change that has been proposed as a substrate of long-term memory is related to changes in dendritic spine density (Horner, 1993; O'Malley et al., 2000; Leuner et al., 2003). There is also a growing body of evidence that strongly suggests that acquisition of novel information induces the development of dendritic spines in the hippocampus in a variety of learning tasks (Mahajan and Desiraju, 1988; Horner, 1993; Moser et al., 1994; Rusakov et al., 1997; O'Malley et al., 2000; Leuner et al., 2003; Restivo et al., 2009), including the one-trial step-through IA task used in the present study (O'Malley et al., 1998). In this case, hippocampal dentate spine density was increased 6 h after training and returned to basal levels at 72 h after training.

Not only is the hippocampus involved in acquisition of conditioned responses, but also in the process of extinction, i.e., in the learned diminution of the conditioned responses following withholding of the unconditioned or reinforcing stimuli (Szapiro et al., 2003; Vianna et al., 2003; Dillon et al., 2008). However, there is a conspicuous lack of information regarding spine formation in this type of learning, and still less regarding extinction of enhanced learning.

The aim of this work was to determine if extinction of an IA response that had been acquired with low, medium, and high (enhanced training) levels of foot-shock promotes spinogenesis in CA1 pyramidal neurons of the hippocampus. If such were the case, then it might represent a possible mechanism for the protective effect of enhanced learning on memory. We now report that IA induces changes in spine density in CA1 pyramidal neurons

of the hippocampus which are dependent on the strength of training.

MATERIALS AND METHODS

ANIMALS

All experimental procedures were approved by the Animal Ethics Committee of Instituto de Neurobiología, Universidad Nacional Autónoma de México and were in compliance with the NIH Guidelines for the Care and Use of Laboratory Animals. The subjects were naive male Wistar rats (250–350 g), maintained in a room with a 12 h/12 h light-dark cycle (lights on at 7:00 h), housed individually in acrylic cages with food and tap water *ad libitum*.

Since the main goal of this work was to determine if extinction after enhanced training of IA induces changes in spine density in the hippocampus as compared to regular training, it was necessary to operationally define "enhanced training." One way to establish such a definition was to test the effects of increasing foot-shock intensities upon resistance to extinction, which is a progressive decline in the magnitude of a conditioned response that is no longer reinforced. It is used as a measure of strength of learning, since resistance to extinction is stronger when the learning experience is also stronger. Therefore, in this experiment, independent groups of rats were given one of three different foot-shock intensities during training in order to find out whether in our experimental conditions a relatively high foot-shock intensity produces stronger resistance to extinction as compared with lower foot-shock intensities. There were 10 rats in each group. The brains of four animals of each group were used to analyze to the density of dendritic spines in the CA1 field of the hippocampus.

APPARATUS

The apparatus is an alley with two distinct compartments of the same size (30 × 30× 30 cm), separated by a guillotine door. The safe compartment had walls and lid of red-colored acrylic with a floor of stainless steel bars (6 mm in diameter, separated by 9 mm); it was illuminated by a 10W light bulb located in the center of its lid. The other non-illuminated compartment had front and back walls and floor made of stainless steel plates with its end walls and lid constructed of red-colored acrylic. The walls and floor were shaped as a trough, 20 cm wide at the top and 8 cm wide at the bottom. In the middle of the floor, a 1.5 cm slot separated the two stainless steel plates that make up the walls and floor. When in this compartment, the rats were in contact with both plates through which foot-shock was delivered. The apparatus was cleansed with 10% alcohol and rinsed with tap water before and after each rat occupied it. A square-pulse stimulator (Grass model No. S-48), in series with a constant current unit (Grass model No. CCU-1A) generated the foot-shock. Shock delivery and measurement of latencies to cross from one compartment to the other one were accomplished by use of automated equipment. The apparatus was located inside a dark, sound-proof room provided with background masking noise.

HANDLING

Before the behavioral experiments began each animal was handled by the experimenter. This consisted of gently touching and holding the rat with both hands using gloves for approximately 5 min for 3 consecutive days. All behavioral observations were carried out between 9:00 h and 13:00 h.

TRAINING AND EXTINCTION TESTING

On the day of training each animal was put inside the safe compartment; 10 s later the door dividing the two compartments was opened and the latency to cross to the other compartment was measured (training latency). When the animals crossed to this compartment the door was closed and a foot-shock (a train of 50 ms square pulses at 10 Hz) of 1.0, 2.0, or 3.0 mA was delivered. Five seconds later the door was reopened allowing the animal to escape to the safe compartment and the stimulator was turned off; this latency was also measured (escape latency). After 30 s in the safe compartment the animals were put back in their home cages. Starting 24 h after training, extinction of the task was measured on six consecutive days. To this end, the same procedure of training was followed except that the foot-shock was omitted; if the animal did not cross to the second compartment within 600 s a retention latency score of 600 was assigned and the session ended. After the last extinction session, four animals from each group were randomly selected to perform the histological analyses described below. Two control groups were added: one of them was subjected to the same training procedure, except that the foot-shock was not administered (0 mA group; $n = 3$). In the case of this group, once they had crossed to the darker compartment, the door was re-opened after 5 s, and if they did not return to the safe compartment within an additional 5 s, they were gently pushed back into it. Twenty-four hours later, and for six consecutive days, latency to enter the dark compartment was also measured. The animals of the second control group ($n = 3$) were kept under identical living conditions as those of the animals used for the behavioral study, but they never left the bioterium, except for sacrificing (Bio group).

RAPID GOLGI IMPREGNATION

Right after the sixth test of extinction the selected animals that had been trained, together with those of the 0 mA and Bio groups, were anesthetized with xylazine/ketamine and perfused transcardially with a buffered solution of 10% formalin (pH 7.4). After 24 h the brains were removed from the skull, and one 4 mm block containing the dorsal hippocampus (Bregma −2.8 to −4.2; Paxinos and Watson, 2005) was obtained from each animal. Each block of hippocampus was impregnated using the rapid Golgi technique, as modified by Diaz-Cintra et al. (1981). After 12 days, each block was transferred to a solution of 0.75% silver nitrate in double-distilled water for 12 h, washed in 50% alcohol, embedded in low-viscosity nitrocellulose, and cut along the frontal plane at a thickness of 120 μm. Each section was collected in 70% alcohol, dehydrated, and mounted in Entellan medium. Each slide had 16 sections of dorsal hippocampus and was assigned a random number to ensure that observers were blind to the experimental conditions.

MORPHOMETRIC ANALYSIS

Dendritic spine counts were performed on five complete, well-impregnated neurons of the CA1 dorsal hippocampus of each

rat. This analysis was carried out with an Optiphot-2 Nikon microscope using a 100× objective (Plan-Apochromat, NA 0.8) and an optically calibrated reticule on the ocular 10× eyepiece. Photomicrographs were assembled from images obtained at different focal planes and reconstructed using Helicon Focus 5.1 software (LTD 2010).

The apical dendrites were divided into proximal, medial, and distal segments, where commissural, Schaffer collaterals, and perforant path fibers arrive, respectively (Amaral and Witter, 1989; Yeckel and Berger, 1990; Dudman et al., 2007). We counted the number of spines along a length of 25 μm of three secondary branches of each segment. Thus, three counts per segment were obtained from each neuron. We chose to analyze secondary branches, and not the main dendritic shaft, because the proximal segment of the main apical dendrite is devoid of spines within the first 100 μm from the soma (Megias et al., 2001; present inspection), and, obviously, no meaningful comparisons could have been made if the proximal segment of the main dendrite would have been taken into account. Dendritic spine density was calculated as the number of dendritic spines divided by the 25 μm of the dendritic branch that was analyzed.

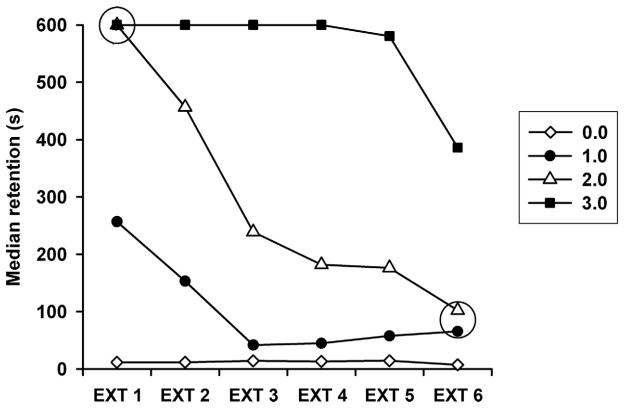

FIGURE 1 | Median retention latencies across the daily extinction sessions (EXT1–EXT6) displayed by the groups that had been trained in inhibitory avoidance with 1.0, 2.0, or 3.0 mA. Also shown is the step-through latency of the 0 mA (control) group. On every extinction session retention scores of each group differed significantly from the latency scores of the other groups, except for the two points within the open circles.

STATISTICAL ANALYSES

Because the measurement of retention was truncated at 600 s, nonparametric statistics were used in analyzing the behavioral results. Independent Kruskal–Wallis analyses of variance were computed for acquisition and escape latencies, and for retention latencies of each of the extinction sessions. When appropriate, the Mann–Whitney *U*-test was used to make comparisons between any two groups.

Independent One-Way ANOVAs were used to analyse spine density in each of the three segments of the apical dendrite. This was done because their different innervations (proximal, medial, and distal segments receive commissural, Schaffer collaterals and perforant path fibers, respectively) indicates that these segments are not homogenous neuronal elements but, rather, independent functional compartments. When the F-ratios were significant, Duncan's Multiple Range Test was used to determine potential statistical differences of pair-wise comparisons.

RESULTS

BEHAVIOR

Training and escape latencies. The Kruskal–Wallis test revealed that there were no significant differences among the groups regarding training latencies ($H(3) = 6.354$, $p = 0.096$). Median training latencies of the 0.0, 1.0, 2.0, and 3.0 mA groups were: 13.0, 24.56, 18.13, and 23.1 s, respectively. Because the "escape" latency of the 0 mA group was not shock-motivated, in every rat this latency was higher than the median escape latency of the foot-shocked groups, as expected; for this reason only the 1.0, 2.0, and 3.0 mA groups were included in the Kruskal–Wallis analysis of escape latencies, which yielded non-significant differenced among them ($H(2) = 0.467$, $p = 0.792$). Escape latencies of the latter groups were: 1.8, 2.2, and 2.1 s, respectively.

Figure 1 depicts the retention of the IA behavior of each group during the 6 days of extinction. A very clear differential pattern of resistance to extinction was produced by the various foot-shock intensities used during training. The Kruskal–Wallis test revealed that there were highly significant differences among the groups on each of the six extinction sessions (p's < 0.001 for each session). As anticipated, the median latency scores of the 0 mA group were very low, always below 15 s, and they differed from those of the other groups in each session (p's ranging from 0.05 to 0.0001). At the other end, the 3.0 mA group displayed near perfect retention latencies on the first five extinction sessions, and a very high score on the sixth session, well above the other groups. Except for the first session, where its retention score did not differ from that of the 2.0 mA group, the 3.0 mA group had a significantly higher retention score than the other groups (p's ranging from 0.01 to 0.0001) across the six sessions. The score of the 2.0 mA group was significantly higher than that of the 1.0 mA group through the first five sessions (p's between 0.05 and 0.01), and in the sixth session, their performances were quite similar ($p = 0.684$).

HISTOLOGY

Figure 2 shows an example of a Golgi-stained pyramidal neuron, and magnified distal, middle and proximal segments where the number of spines was tallied along a length of 25 μm. The statistical analyses revealed that there were highly significant differences in spine density among the groups in the proximal ($F_{(4,266)} = 9.635$, $p = 0.0001$), medial ($F_{(4,266)} = 26.978$, $p = 0.0001$), and distal ($F_{(4,266)} = 20.245$, $p = 0.0001$) segments. The Duncan's Multiple Range Test showed that there were no significant differences in spine density between the Bio and the 0 mA groups in any of the segments (**Figure 3**). Regarding the proximal segment, spine density of the Bio and the 0 mA groups was significantly higher than that of the 1.0, 2.0, and 3.0 mA groups; spine density was not significantly different among the latter three groups. In the medial segment, the Bio and the 0 mA groups also had a higher spine density than the 1.0, 2.0, and 3.0 mA groups; in turn,

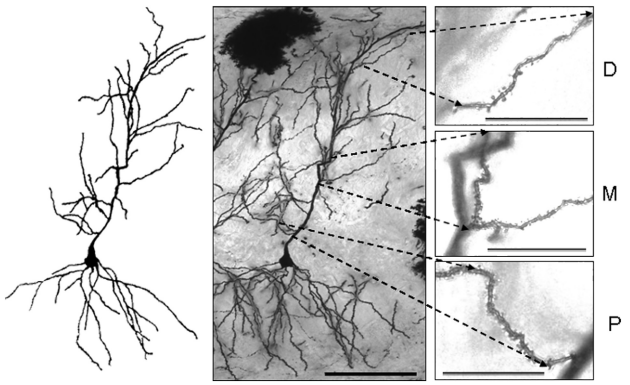

FIGURE 2 | Representative example of CA1 pyramidal cells stained with the rapid-Golgi technique (middle figure), and a camera lucida drawing of it (left figure). Insets on the right hand side show enlarged images of distal (D), medial (M), and proximal (P) segments of the apical dendrite. Dendritic spines could be easily seen in each image with examination at different focal planes. Scale bars: middle figure (20×) = 100 μm; insets (100×) = 25 μm.

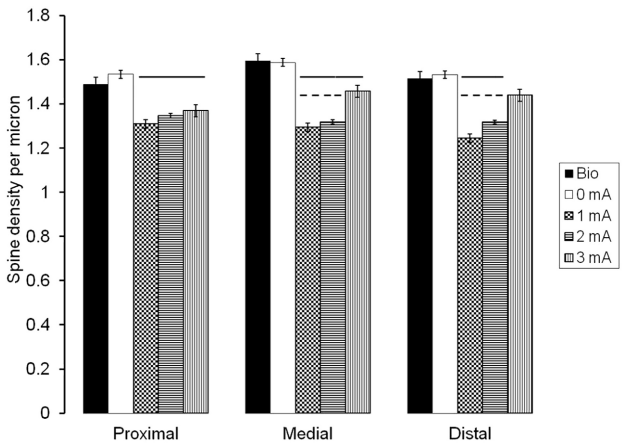

FIGURE 3 | Mean spine density values (spines per micrometer) in each of the three segments of the CA1 apical dendrite, found after six extinction sessions of animals trained in inhibitory avoidance with 0, 1.0, 2.0, or 3.0 mA, and of a control group that never left the bioterium, except for sacrificing (Bio). Solid lines over bars represent significant differences ($p < 0.001$) as compared to Bio and 0 mA groups; broken lines over bars represent significant differences ($p < 0.001$) as compared to the 3.0 mA group.

the 3.0 mA group showed a higher spine density than the 1.0 and 2.0 mA groups. Finally, in the distal segment, no significant differences among the Bio, 0 mA, and 3.0 mA groups were found; each of these groups differed significantly from the 1.0 and 2.0 mA groups.

Percent change in spine density, relative to the Bio control group is depicted in **Figure 4**. It can be clearly seen the spine pruning in the three segments of the apical CA1 dendrite of all the trained groups, except that the high foot-shock group maintained normal spine density in the distal segment.

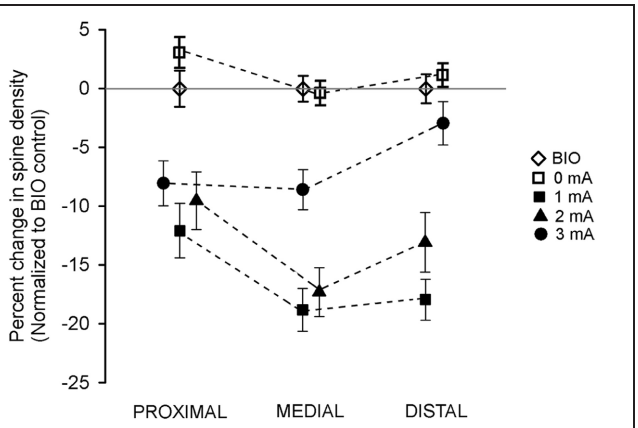

FIGURE 4 | Effects of intensity of inhibitory avoidance training on percentage changes in spine density found after six extinction sessions, normalized to the BIO control group, in each of the three segments of the apical dendrites of neurons of the CA1 dorsal hippocampus of the rat.

DISCUSSION

This is the first study, to our knowledge, to examine spine plasticity in the hippocampus after a procedure of extinction.

Two main results were obtained. First, as expected, the higher foot-shock intensity induced better learning, i. e., the 3.0 mA group showed significantly higher resistance to extinction, as it performed better than the other groups on the extinction sessions.

The second main finding was that extinction produced pruning of spines in the three segments of the apical dendrite in the groups that had been trained with the lower foot-shock intensities (1.0 and 2.0 mA). Just as interesting was the observation that animals submitted to an enhanced learning experience (3.0 mA group), which produced higher resistance to extinction, showed different spine densities along the length of the apical dendrite: there was pruning in the proximal segment, as was the case for the lower foot-shock groups, a significant increase in spine density in the medial segment, and a spine density that was as high as that of the control groups in the distal segment.

The reduction in spine numbers after extinction was not simply due to exposure to the experimental context nor to habituation to it; this inference can be made because such a reduction in spines was not observed in the group that was "trained" with 0 mA and exposed to the same context for the same number of sessions as the trained, foot-shocked groups.

The presumption that synaptic changes underlie memory storage is not new. On the first half of the twentieth century Pavlov (1927) and Konorski (1948) postulated that conditioning was a form of learning different from that engaged in extinction but that, nonetheless, these two learning processes were associated with the making of new synaptic connections. There is a good deal of experimental support for the notion that learning is associated to genesis of dendritic spines (Rusakov et al., 1997; O'Malley et al., 1998, 2000; Leuner et al., 2003; Marrone, 2007), but it is difficult to find reports dealing with spine dynamics and extinction.

Our results point to one probable mechanism of extinction and of the protective effect of enhanced learning against

interference with long-term memory formation. It was quite clear that those groups that actually showed extinction (1.0 and 2.0 mA groups; **Figure 1**) also presented a significant degree of spine pruning along the three segments of the CA1 apical dendrites (**Figure 3**). In this case, the plastic change induced by extinction was in the form of a reduction of synaptic contacts in the apical dendrite, as can be implied by the reduction of spines, and suggests that extinction is linked to a decrement in excitatory inputs at the postsynaptic site. This finding goes counter to the hypotheses proposed by Pavlov (1927) and Konorski (1948), and clearly shows that the mechanisms underlying the new learned response (extinction) are quite different from that those mediating the original learning of the IA response, where spine density has been found to increase significantly (O'Malley et al., 1998).

On the other hand, the group that was trained with the highest foot-shock intensity (3.0 mA), showed perfect retention of the conditioned response through the first four sessions of extinction, and it was on the fifth session that this response only begun to decline. Thus, it is obvious that the behavior of this group reflected the effects of enhanced training. This heightened resistance to extinction was accompanied by a differential pattern of spine modifications through the length of the CA1 apical dendrite (**Figure 4**). Just as in the case of training with the lower foot-shock intensities, this group showed pruning in the proximal and medial segments of the apical dendrite. However, in the distal segment spine count was not different from the control groups. This interesting result may indicate that the maintenance of the conditioned response of this group, in spite of having been submitted to the process of extinction, may be due, at least in part, to the maintenance of synaptic connections mediated by these dendritic spines. The main afferent fibers of the distal segment of the apical dendrite, the perforant path, arise from the multimodal entorhinal cortex. It is tempting to speculate that this pathway conveys excitatory signals produced by the enhanced learning conditions, thus halting the natural pruning induced by the extinction of the conditioned response.

In a recent and stimulating article, Vetere et al. (2011) studied dendritic spine dynamics after long-term retention of contextual fear conditioning. A significant increase in the density and size of spines was found in the apical dendrites of the anterior cingulated and infralimbic cortices. Interestingly, after extinction of the conditioned response spine density returned to baseline but the proportion of large spines did not in the anterior cingulated cortex; the opposite outcome was seen in the infralimbic cortex. These authors concluded that fluctuations in spine density and spine shape occur in cortical regions during both the formation and the extinction of contextual fear memories. On the other hand, we found that extinction was accompanied by pruning of dendritic spines along the length of the apical dendrites of hippocampal CA1 cells of rats that had been trained in IA with relatively low foot-shocks, and no change in spine density in the distal dendrite when a high foot-shock was administered. These dissimilar findings show that extinction learning represents a complex phenomenon where different processes take place in different brain regions, which may be dependent upon the type of behavior that is being extinguished.

It is worth mentioning that one distinctive feature of post-traumatic stress disorder (PTSD) is the formation of a strong memory that is highly resistant to extinction (Armario et al., 2008), akin to the memory formed after the enhanced learning of IA described in the present work. Thus, this behavioral model might be useful in the study of the neurobiology of PTSD, and one is tempted to hypothesize that resistance to extinction seen in this pathology could also be mediated by the durability of dendritic spines in the apical dendrites of the hippocampal pyramidal neurons.

To conclude, training of IA with a foot-shock of relatively high intensity (3.0 mA) produced enhanced learning, as shown by the strong resistance to extinction, as compared to the performance of the groups that had been trained with lower foot-shock intensities. After testing under a protocol to produce extinction, there was pruning of dendritic spines along the length of the apical dendrites in neurons of the CA1 field of the hippocampus in the animals that were trained with the lower foot-shock intensities (1.0 and 2.0 mA); these animals showed weak resistance to extinction. The group with the strong resistance to extinction also showed pruning of dendritic spines, but only in the proximal and medial segments of the apical dendrite; normal spine counts were found in the distal segment. Behavioral extinction, evidenced by the low retention scores, may be mediated, in part, by a plastic change in the form of a reduction of synaptic contacts in the apical dendrite, while maintenance of the conditioned response after the extinction protocol may be due, at least in part, to the maintenance of synaptic connections in the distal segment of the apical dendrite.

Further research is needed to put to the experimental test the various interpretations to the results offered in this work.

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The endocannabinoid transport inhibitor AM404 differentially modulates recognition memory in rats depending on environmental aversiveness

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Cannabinoid compounds may influence both emotional and cognitive processes depending on the level of environmental aversiveness at the time of drug administration. However, the mechanisms responsible for these responses remain to be elucidated. The present experiments investigated the effects induced by the endocannabinoid transport inhibitor AM404 (0.5–5 mg/kg, i.p.) on both emotional and cognitive performances of rats tested in a Spatial Open Field task and subjected to different experimental settings, named *High Arousal (HA)* and *Low Arousal (LA)* conditions. The two different experimental conditions influenced emotional reactivity independently of drug administration. Indeed, vehicle-treated rats exposed to the *LA* condition spent more time in the center of the arena than vehicle-treated rats exposed to the *HA* context. Conversely, the different arousal conditions did not affect the cognitive performances of vehicle-treated animals such as the capability to discriminate a spatial displacement of the objects or an object substitution. AM404 administration did not alter locomotor activity or emotional behavior of animals exposed to both environmental conditions. Interestingly, AM404 administration influenced the cognitive parameters depending on the level of emotional arousal: it impaired the capability of rats exposed to the *HA* condition to recognize a novel object while it did not induce any impairing effect in rats exposed to the *LA* condition. These findings suggest that drugs enhancing endocannabinoid signaling induce different effects on recognition memory performance depending on the level of emotional arousal induced by the environmental conditions.

Keywords: cannabinoid system, endocannabinoids, emotionality, short-term memory, cognition

INTRODUCTION

The endocannabinoid system is a crucial regulator of central nervous system (CNS) function (Cravatt et al., 1996; Di Marzo and Matias, 2005; Pacher et al., 2006; Trezza et al., 2008b; Campolongo et al., 2009b,c, 2011; Bisogno and Di Marzo, 2010; Hill and McEwen, 2010). Endocannabinoids are released from post-synaptic neurons in an activity-dependent manner, travel retrogradely through the synaptic cleft and activate pre-synaptic cannabinoid type 1 receptors (CB1), thus suppressing neurotransmitter release from axon terminals (Wilson and Nicoll, 2002). Among the endogenous cannabimimetic signaling molecules, anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) stand out as the first identified and most intensively studied (Ueda et al., 1995, 2011; Di Marzo, 1998; Piomelli, 2003; Waku, 2006). Receptor activation by endocannabinoids ends by the removal from the synaptic cleft operated by a transport system present in neural and non-neural cells (Di Marzo et al., 1994; Beltramo et al., 1997; Hillard et al., 1997) followed by hydrolysis operated by fatty-acid amide hydrolase (FAAH, that hydrolyzes anandamide) or monoacylglycerol lipase (MAGL, that cleaves 2-AG) (Desarnaud et al., 1995; Hillard et al., 1995; Ueda et al., 1995; Cravatt et al., 1996). Interestingly, while the endocannabinoid hydrolyzing enzymes have been fully identified and cloned, the functional properties of the putative transporter have been only partially characterized (Hillard and Jarrahian, 2003; Yates and Barker, 2009; Fu et al., 2011) and its molecular identity remains still unknown.

CB1 receptor is crucially involved in neural plasticity mechanisms related to the processing, consolidation, and extinction of emotionally salient cognitive events (Marsicano et al., 2002; Laviolette and Grace, 2006a,b; Campolongo et al., 2009a,b; Mackowiak et al., 2009; Abush and Akirav, 2010; Akirav, 2011; Hauer et al., 2011). This fits well with the notion that CB1 receptors are highly expressed in brain structures including the basolateral amygdala (BLA), the medial prefrontal cortex (mPFC) and the hippocampus (Breivogel and Childers, 1998; Mackie, 2005; Katona, 2009), strictly associated with both cognitive and

emotional processes (Laviolette and Grace, 2006a; Viveros et al., 2007; McLaughlin and Gobbi, 2011; Tan et al., 2011).

Animal studies have demonstrated that the endocannabinoid system modulates recognition memory by altering the mechanisms responsible for this process within the hippocampus and selectively affecting the encoding stage (Barna et al., 2007). Moreover, the important involvement of other structures, for instance the amygdala, in the modulation of memory consolidation and extinction for emotional events has been firmly established (McGaugh, 2000; Vianna et al., 2004; Clarke et al., 2008; de Oliveira Alvares et al., 2008, 2010; Campolongo et al., 2009b; Ganon-Elazar and Akirav, 2009; Manwell et al., 2009; Roozendaal and McGaugh, 2011). In line with the widespread distribution of CB1 receptors throughout the limbic system, it has been extensively demonstrated that cannabinoid compounds also induce diverse effects on anxiety- and fear-related behaviors (Trezza et al., 2008a, 2012; Micale et al., 2009; Moreira and Wotjak, 2010; Terzian et al., 2011). Interestingly, cannabinoid effects on emotionality are biphasic, as it is also reported by cannabis abusers (Fant et al., 1998; Hall and Solowij, 1998; Bolla et al., 2002; Curran et al., 2002). The classical explanation to this phenomenon is often provided by the use of different doses of cannabinoid drugs, with low doses generally inducing anxiolytic-like effects and high doses often causing the opposite. A new and appealing explanation to this phenomenon is now emerging, underlying that these opposite effects may also depend on previous experiences, the context of use and the level of emotional arousal at the time of drug administration/consumption (Akirav, 2011; Sciolino et al., 2011). Drugs that interfere with endocannabinoid degradation increase ongoing endocannabinoid signaling in a temporarily and spatially restricted manner (Janero et al., 2009). However, preclinical evidence has shown that indirect cannabinoid agonists can also induce biphasic effects on behavior, depending on the emotional state of the subject. For instance, it has been recently demonstrated that the FAAH inhibitor URB597 does not affect anxiety under mildly stressful circumstances but has robust anxiolytic-like effects in highly aversive testing conditions (Haller et al., 2009). This finding leaves open the possibility that inhibitors of endocannabinoid transport, which prolong endocannabinoid actions by preventing endocannabinoid access to intracellular hydrolyzing enzymes (Beltramo et al., 1997; Kathuria et al., 2003), may influence both emotional and cognitive processes depending on the level of environmental aversiveness at the time of drug administration.

To address this issue, in the present study we investigated the effect of the prototypical endocannabinoid transport inhibitor, AM404 in a non-aversive task, the Spatial Open Field test under two experimental conditions differing by the level of emotional arousal at the time of testing. The Spatial Open Field task has been extensively used (Poucet et al., 1986; Thinus-Blanc et al., 1987; Poucet, 1989, 1993; Ricceri et al., 1999, 2002; Scattoni et al., 2004; de Bartolo et al., 2010) and permits to assess both emotional and cognitive parameters, in terms of reactivity to a spatial or an object novelty, by exploiting the natural propensity of rodents to explore the environment. The *High Arousal condition* (HA) was obtained by testing rats in an empty arena under white light illumination without previous handling, while the *Low Arousal condition* (LA) was obtained by extensively handling the animals before testing in an arena with the ground loaded with familiar bedding, under a dim red lighted room.

By manipulating the experimental conditions and the tone of endogenous cannabinoids, this study may help to explain how the interaction between endocannabinoids and environment could influence recognition memory in rats.

MATERIALS AND METHODS

ANIMALS

Male adult Wistar rats (300 g at the time of testing, Charles River Laboratories, Italy) were housed in groups and maintained in a temperature-controlled environment (20 ± 1°C) under a 12 h light/12 h dark cycle (7:00 am to 7:00 pm lights on) with unlimited access to food and water. All procedures involving animal care or treatments were approved by the Italian Ministry of Health and performed in compliance with the guidelines of the US National Institutes of Health (NIH) and the Italian Ministry of Health (D.L. 116/92), the Declaration of Helsinki, the Guide for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council 2004) and the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes.

DRUG TREATMENTS

N-(4-Hydroxyphenyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamide (AM-404, 0.5–1–5 mg/kg), purchased from Tocris Bioscience (UK), was dissolved in a vehicle containing 10% polyethylene glycol, 10% Tween-80, and 80% saline. Drug solutions were freshly prepared before each experiment and administered by intraperitoneal injection in a volume of 1 ml/kg 15 min before the beginning of the task.

SPATIAL OPEN FIELD PROCEDURES

The apparatus consisted in an open-field arena made of black Plexiglas (80 × 80 × 60 cm) surrounded with a visually uniform environment. A video camera above the field was connected to a video recorder. Experiments were performed between 10.00 am and 2.00 pm.

To assess the effect of the exogenous manipulation of the endocannabinoid tone on short-term memory performance, the test schedule consisted of a single task composed by six 5-min consecutive sessions taking place during the same day, separated by 3-min delays during which the subjects were returned to their home cage (**Figure 1**). During session 1, each rat was placed into the center of the empty arena to allow it to become familiar with the apparatus and to record baseline levels of locomotor and exploratory activity. Starting from session 2, three different objects were simultaneously present in the open field: Object A, a dark metal parallelepiped (4 cm high × 13 cm wide × 9 cm long); Object B, a transparent Plexiglas cube with holes regularly distributed on the sides (height = 10 cm); Object C, a gray plastic square (10 × 10 × 10 cm) with a central triangle forming a 90° angle. During sessions 2–3, the A, B, and C objects were placed in the arena. In session 4, the spatial test session, the configuration was changed by moving two objects: object B replaced object A which was itself displaced at the periphery of the apparatus.

In session 5, the configuration of the objects was unchanged to let the rats habituate to the new arrangement of the objects. In the last session (session 6) one of the familiar, non-displaced objects (object C) was replaced by a new object (object D), which consisted of a black-and-white plastic cylinder, height = 13 cm; diameter = 6 cm (**Figure 1**).

We exposed the rats to two experimental conditions, named HA and LA conditions. In the HA condition (Experiment 1), the test was performed under normal light (30–40 lux), rats were not handled and tested in an empty arena (no bedding). In the LA condition (Experiment 2), the test was performed under dim red light (2 lux) condition, rats were extensively habituated to the experimenter and to the injection procedure for one week before the experiment (every day, 1 min per each rat) and tested in an arena with the ground loaded with familiar bedding.

STATISTICAL ANALYSIS

Data collection was performed from the same observer who was unaware of animal treatment using the Observer XT software (Noldus, Netherland). During the first session, frequency and/or duration of the following responses were measured: crossings, rearings, and time spent in the center of the apparatus. From sessions 2–6, object exploration was measured as total time spent by the animal in contact with an object (1 s as minimal contact was considered) throughout all sessions 2–6.

The total time spent by rats investigating all objects throughout all sessions has been considered as an indicator of general investigative activity. A contact was defined as the subject's snout actually touching an object. In session 4, the spatial arrangement of the objects was modified and response to spatial change was assessed by comparing the mean time spent in contact with both Displaced (DO) and Non-Displaced (NDO) Objects in session 4 minus the mean time spent in contact with the same object in session 3. A discrimination index of the response to the spatial change was obtained by subtracting the NDO value to DO value. Finally, the response to the non-spatial novelty was assessed by comparing mean time in contact with the Substituted Object (SO, unfamiliar) and Non-Substituted Objects (NSO, familiar) in session 6 minus the mean time spent with objects located in the corresponding position in session 5. A discrimination index of the response to the non-spatial novelty was obtained by subtracting the NSO value to SO value. Unpaired t-test was used to compare the behavioral performance of vehicle groups. One-sample t-tests were used to determine whether the discrimination index was different from zero. Treatment (AM404) effects were analyzed by One-Way analysis of variance (ANOVA) or ANOVA for repeated measures (when appropriate), followed by Tukey's *post-hoc* comparison tests. A probability level of < 0.05 was accepted as statistically significant.

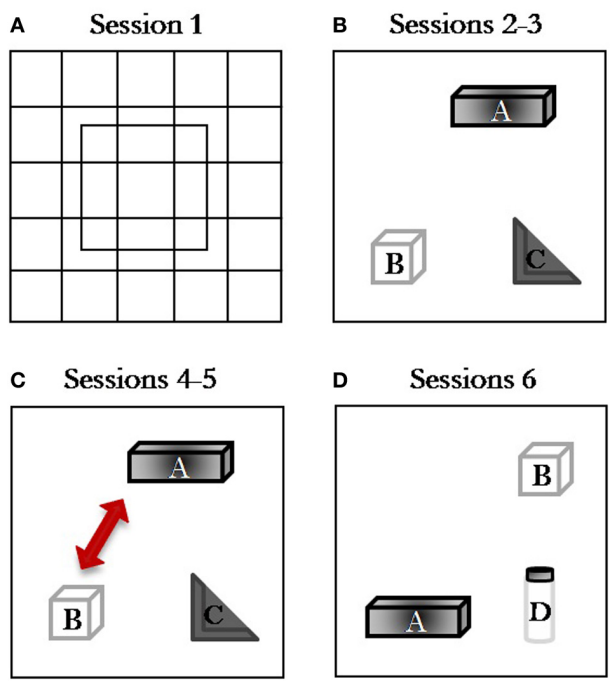

FIGURE 1 | Spatial Open-Field procedure. Schematic diagram representing the object configuration in the Spatial Open-Field test: **(A)** session 1, open field without objects; **(B)** session 2, habituation session with three stable objects; **(C)** sessions 3–4, spatial change discrimination sessions where object B displaced object A (session 4); **(D)** session 5–6, object novelty sessions where object D replaced object C (session 6).

RESULTS

DIFFERENT AROUSAL CONDITIONS INFLUENCED EMOTIONAL BEHAVIOR AND OBJECT EXPLORATION BUT DID NOT ALTER COGNITIVE PERFORMANCES OF VEHICLE-TREATED ANIMALS

Unpaired t-test showed that the different arousal context did not affect locomotor activity of the vehicle groups. Both crossing (**Figure 2A**) and rearing (**Figure 2B**) frequencies did not statistically differ between the two groups ($t = -0.66$; $p = 0.52$; $t = 1.09$; $p = 0.29$, respectively). However, unpaired t-test showed that the different arousal conditions influenced the emotional behavior of vehicle-treated animals exposed to the different experimental contexts. Rats treated with vehicle and exposed to a *High Arousal* condition (HA group) spent less time in the center of the arena than vehicle-treated rats exposed to a *Low Arousal* context (LA group) ($t = -4.11$; $p = 0.0005$, **Figure 2C**).

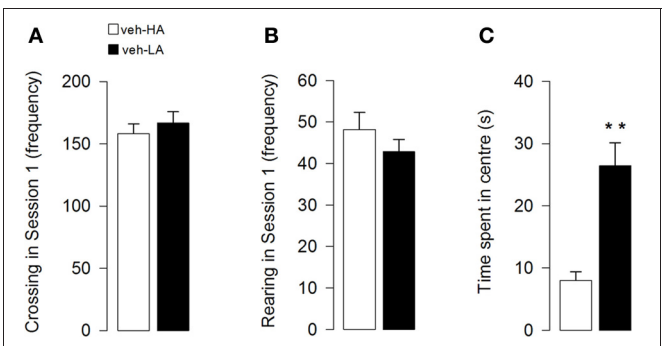

FIGURE 2 | Effects induced by different arousal conditions on locomotor activity and emotional behavior of vehicle-treated rats. Locomotor activity: number of crossing **(A)** and rearing **(B)** in session 1. Emotional behavior: time spent in the center of the arena in session 1 **(C)**. $^{**}P < 0.01$. Data are expressed as mean ± SEM. (High Arousal: HA $n = 10$; Low Arousal: LA $n = 15$).

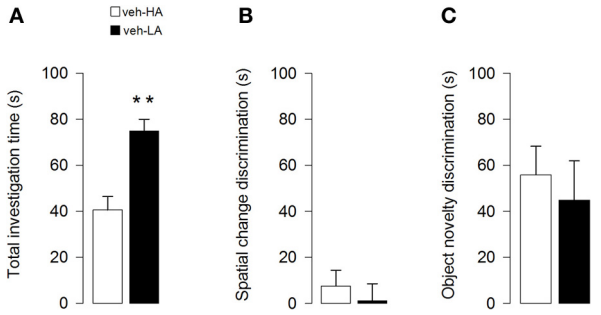

FIGURE 3 | Effects induced by different arousal conditions on object investigation and cognitive performances of vehicle-treated rats. Total investigation time of all objects through sessions **(A)** spatial change discrimination **(B)** and object novelty discrimination **(C)**.$^{**}P < 0.01$. Data are expressed as mean ± SEM. (High Arousal: HA $n = 10$; Low Arousal: LA $n = 15$).

Unpaired t-test showed that rats treated with vehicle and exposed to a HA context spent less time investigating objects than vehicle-treated rats exposed to a LA context ($t = -4.41$; $p < 0.0001$, **Figure 3A**). Additionally, unpaired t-test showed that both vehicle groups did not differ in the discrimination index for a spatial object displacement in session 4 ($t = 0.60$; $p = 0.55$, **Figure 3B**) and for the substitution of the objects in session 6 ($t = 0.47$; $p = 0.64$, **Figure 3C**). However, One-sample t-tests revealed that while both vehicle groups were able to discriminate the object novelty (veh-HA, $t9 = 4.49$, $P = 0.0015$; veh-LA, $t14 = 2.61$, $P = 0.02$, **Figure 3C**) they did not respond to a spatial rearrangement (veh-HA, $t9 = 1.10$, $P = 0.30$; veh-LA, $t14 = 0.16$, $P = 0.88$, **Figure 3B**).

AM404 ADMINISTRATION DID NOT ALTER LOCOMOTOR ACTIVITY AND EMOTIONAL BEHAVIOR IN RATS EXPOSED TO DIFFERENT AROUSAL CONDITIONS

AM404 administration did not alter locomotor activity of rats exposed to either a HA or LA condition. One–Way ANOVA for crossing (**Figure 4A**) and rearing (**Figure 4B**) frequencies in session 1 for AM404-treated rats exposed to a HA condition did not show a statistically significant difference ($F_{3,\ 36} = 0.60$; $p = 0.62$; $F_{3,\ 36} = 1.44$; $p = 0.25$, respectively). Moreover, One–Way ANOVA for the number of crossings (**Figure 4C**) or rearings (**Figure 4D**) in session 1 did not show a statistically significant difference between vehicle- and AM404-treated rats exposed to a LA condition ($F_{3,\ 50} = 0.97$; $p = 0.42$; $F_{3,\ 50} = 2.21$; $p = 0.10$, respectively).

AM404 administration did not affect emotional reactivity in rats exposed to either a HA or LA condition. Indeed, One-Way ANOVA showed that vehicle- and AM404-treated rats did not differ for the time spent in the center of the arena in session 1 (HA condition: $F_{3,\ 36} = 1.25$; $p = 0.31$; **Figure 5A**; LA condition: $F_{3,\ 50} = 1.18$; $p = 0.33$; **Figure 5B**).

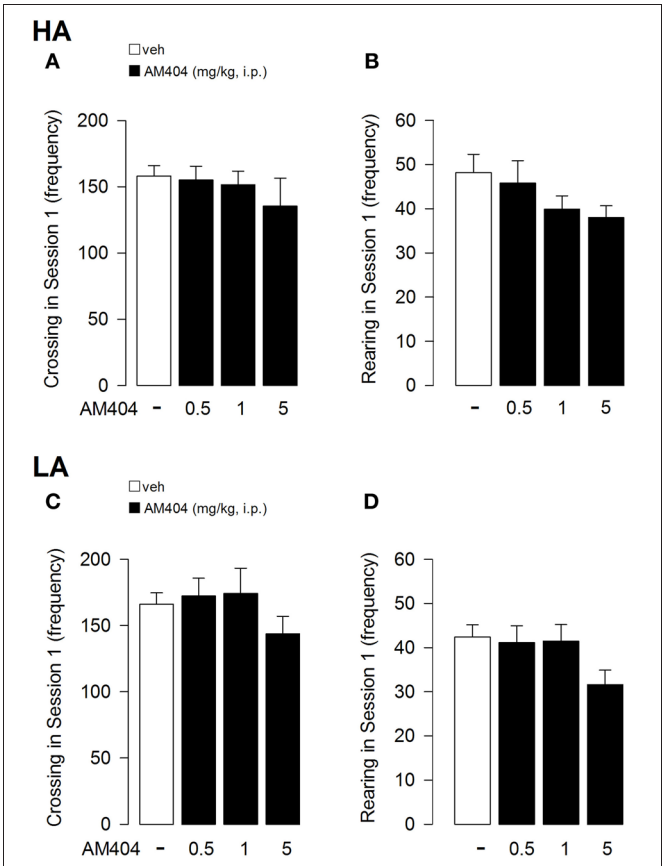

FIGURE 4 | Effects of AM404 administration on locomotor activity in rats exposed to high arousal (HA) or low arousal (LA) conditions. Number of crossing **(A)** and rearing **(B)** of rats exposed to HA or LA conditions **(C, D,** respectively**)** in session 1. Data are expressed as mean ± SEM. (HA: veh $n = 10$, 0.5 mg/kg $n = 11$, 1 mg/kg $n = 10$, 5 mg/kg $n = 9$; LA: veh $n = 15$, 0.5 mg/kg $n = 12$, 1 mg/kg $n = 14$, 5 mg/kg $n = 13$).

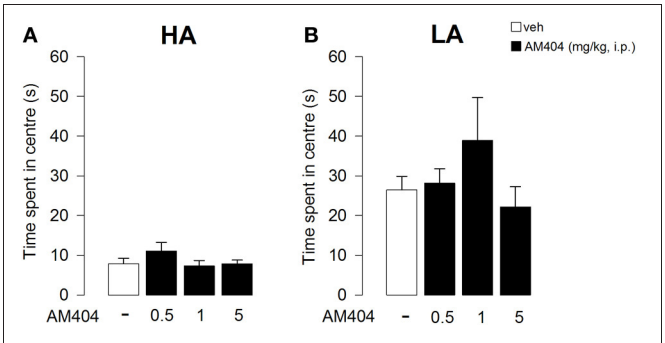

FIGURE 5 | Effects of AM404 administration on emotional behavior in rats exposed to high arousal (HA) or low arousal (LA) conditions. Time spent in the center of the arena by rats exposed to HA **(A)** or LA conditions **(B)** in session 1. Data are expressed as mean ± SEM. (HA: veh $n = 10$, 0.5 mg/kg $n = 11$, 1 mg/kg $n = 10$, 5 mg/kg $n = 9$; LA: veh $n = 15$, 0.5 mg/kg $n = 12$, 1 mg/kg $n = 14$, 5 mg/kg $n = 13$).

AM404 ADMINISTRATION INFLUENCED OBJECT EXPLORATION DEPENDING ON THE DIFFERENT AROUSAL CONDITION

The time spent in contact with objects throughout sessions 2–6 was analyzed with a mixed model-ANOVA taking treatment as one between-subject factor and sessions as one repeated measure factor. The mixed model-ANOVA for rats subjected to the HA condition gave the following differences: treatment $F_{3,\ 33} = 2.72$; $p = 0.02$; sessions $F_{4,\ 132} = 39.78$; $p < 0.0001$,

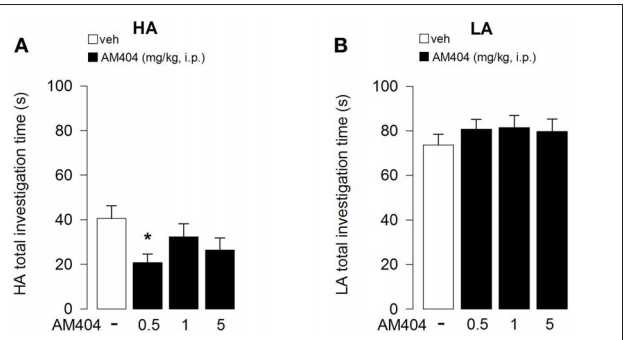

FIGURE 6 | Effects of AM404 administration on object investigation in rats exposed to high arousal (HA) or low arousal (LA) conditions. Total investigation time of all objects by rats exposed to HA **(A)** or LA **(B)** conditions through sessions 2–6. $*P < 0.05$. Data are expressed as mean ± SEM. (HA: veh $n = 10$, 0.5 mg/kg $n = 11$, 1 mg/kg $n = 10$, 5 mg/kg $n = 9$; LA: veh $n = 15$, 0.5 mg/kg $n = 12$, 1 mg/kg $n = 14$, 5 mg/kg $n = 13$).

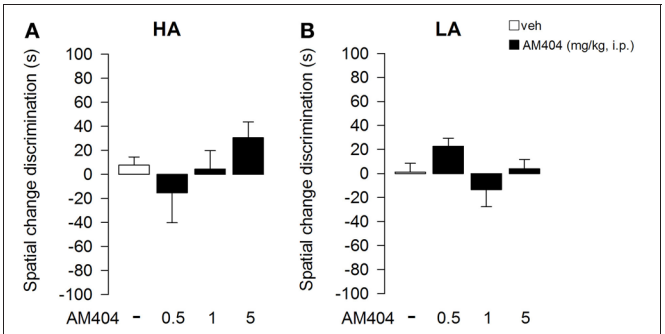

FIGURE 7 | Effects of AM404 administration on spatial change discrimination in rats exposed to high arousal (HA) or low arousal (LA) conditions. Spatial change discrimination index of rats exposed to HA **(A)** or LA **(B)** conditions. Tim spent in contact with Displaced (DO) and Non-Displaced (NDO) Objects in session 4 minus the mean time spent in contact with the same object in session 3. A discrimination index was obtained by subtracting the NDO value to DO value. Data are expressed as mean ± SEM. (HA: veh $n = 10$, 0.5 mg/kg $n = 11$, 1 mg/kg $n = 10$, 5 mg/kg $n = 9$; LA: veh $n = 15$, 0.5 mg/kg $n = 12$, 1 mg/kg $n = 14$, 5 mg/kg $n = 13$).

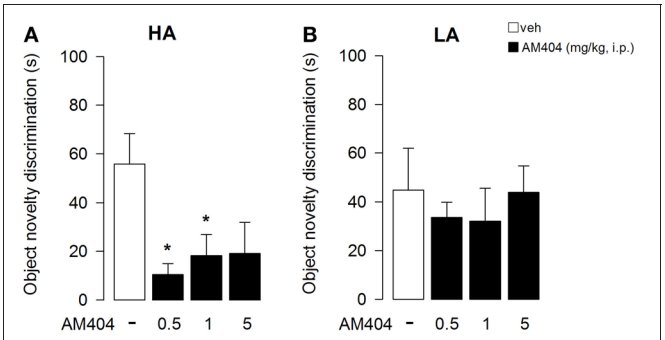

FIGURE 8 | Effects of AM404 administration on object novelty discrimination in rats exposed to high arousal (HA) or low arousal (LA) conditions. Object novelty discrimination index of rats exposed to HA **(A)** or LA **(B)** conditions. Time spent in contact with Substituted Object (SO, unfamiliar) and Non-Substituted Objects (NSO, familiar) in session 6 minus the mean time spent with objects located in the corresponding position in session 5. A discrimination index was obtained by subtracting the NSO value to SO value. $*P < 0.05$. Data are expressed as mean ± SEM. (HA: veh $n = 10$, 0.5 mg/kg $n = 11$, 1 mg/kg $n = 10$, 5 mg/kg $n = 9$; LA: veh $n = 15$, 0.5 mg/kg $n = 12$, 1 mg/kg $n = 14$, 5 mg/kg $n = 13$).

interaction treatment × sessions $F_{12,\ 132} = 1.50$; $p = 0.13$. Since the interaction between treatment and sessions was not statistically significant, for a clearer representation of the results, data are represented in **Figure 6** as the mean time spent in contact with the objects during all sessions. Individual comparisons (Tukey's test) performed on the main effect of treatment, revealed that AM404 (0.5 mg/kg) treated rats spent less time exploring the objects than control animals ($p < 0.05$, **Figure 6A**).

On the other hand, the mixed model-ANOVA for rats subjected to the LA condition revealed only a statistical significant effect for sessions without any statistical significance for either treatment or interaction between treatment and sessions: treatment $F_{3,\ 46} = 0.12$; $p = 0.95$; sessions $F_{4,\ 184} = 20.82$; $p < 0.0001$, interaction treatment × sessions $F_{12,\ 132} = 1.20$; $p = 0.29$ (**Figure 6B**), indicating that AM404 administration did not influence the total object investigation time in rats subjected to the LA arousal condition.

AM404 ADMINISTRATION DID NOT INFLUENCE *SPATIAL CHANGE* DISCRIMINATION WHILE IT ALTERED *OBJECT NOVELTY* RECOGNITION IN RATS EXPOSED TO DIFFERENT AROUSAL CONDITIONS

One–Way ANOVA showed that administration of AM404 did not influence the rat capability to discriminate the object displacement under both the HA (**Figure 7A**) or LA (**Figure 7B**) experimental conditions ($F_{3,\ 36} = 1.176$; $p = 0.34$; $F_{3,\ 50} = 2.24$; $p = 0.095$, respectively). However, One–Way ANOVA showed a statistical significant effect on the capability of the rats to discriminate a novel object under a HA condition ($F_{3,\ 36} = 4.32$; $p = 0.01$; **Figure 8A**). *Post-hoc* comparisons revealed that rats administered with AM404 0.5 and 1 mg/kg were not able to discriminate the new object as vehicle-treated rats did ($p < 0.05$). One–Way ANOVA revealed that AM404 administration to LA exposed rats did not influence the capability of the rats to discriminate the new object compared to the vehicle group ($F_{3,\ 50} = 0.26$; $p = 0.85$; **Figure 8B**).

DISCUSSION

The present findings demonstrate that: (1) different levels of environmental aversiveness strongly influence the emotional reactivity of untreated rats without affecting the cognitive performance in the Spatial Open-Field test; (2) endocannabinoids affect recognition memory of rats in the Spatial Open Field test depending on the level of emotional arousal induced by the environmental conditions.

The Spatial Open-Field is a non-aversive test that permits to assess several behaviors which are indicative of the emotional state of the animal as well as the reactivity to both spatial rearrangement (spatial novelty) or the replacement of one familiar object with a new one (object novelty, as in the classical object recognition task) (Poucet et al., 1986; Thinus-Blanc et al., 1996). This test exploits the natural propensity of rodents to explore the environment without using rewards or punishments. Previous studies have shown that naive rodents respond to a

new spatial displacement or substitution by renewed exploration of the entire environment and/or by selective reinvestigation of the displaced/substituted objects (Poucet et al., 1986; Thinus-Blanc et al., 1987; Poucet, 1989, 1993; Ricceri et al., 1999, 2000, 2002; Scattoni et al., 2004; de Bartolo et al., 2010). The one-day six-session assessment of the task used in our study permits to determine pharmacological effects on short-term memory as well as on emotional reactivity of the subject.

Activation of emotional responses, triggered by stressful stimuli, is crucial in the modulation of contextual learning and memory performances (McGaugh and Roozendaal, 2002; McGaugh, 2004; Morris, 2006; Campolongo et al., 2009b; Hill et al., 2010). There is evidence that behavioral responses to the environmental stimuli are strictly dependent on the emotional reactivity induced by the environment itself (Blanchard et al., 2001; Haller et al., 2009). The environmental–induced arousal is critically involved in assessing the novelty and salience of the external stimuli in terms of relevance for the adaptation and survival (Poucet, 1993; Biegler and Morris, 1996; Breivogel and Childers, 1998). Thus, when compared with a previous experience, a novel information recognized as highly relevant is committed to and stored by the memory (Lemaire et al., 1999). However, the mechanisms underlying the modulation of responsiveness to the environment and its evaluation in evolutionary terms both under LA or HA contexts remain to be elucidated.

Based on previous findings (Szeligo and Leblond, 1977; Sahakian et al., 1982; Morato and Castrechini, 1989; Griebel et al., 1993; Escorihuela et al., 1994; Hall et al., 1998; Varty et al., 2000; Haller et al., 2009), in order to characterize the behavioral responses to different environmental situations, we manipulated the experimental context to create two opposite arousal conditions by using two different protocols: (1) rats either extensively handled or not handled by the experimenter before testing, (2) isolated- or grouped-housed rats; (3) bright or dim red light conditions; (4) without or with familiar bedding during the testing phase for HA or LA conditions, respectively (for a comprehensive description see Materials and Methods). By using these different experimental conditions, we were able to induce a high or a low state in the animal, independently of any drug administration.

To first characterize the behavioral responses of rats to different environmental situations in the Spatial Open Field task, regardless of any drug administration, we analyzed the performance of vehicle-treated rats exposed to a HA or a LA context. The analysis of the first session of the Spatial Open Field task (when no objects were present) showed that locomotor activity was not influenced by the two different arousal conditions, while the different environmental situations influenced the level of emotional reactivity of the animals. Vehicle-treated rats, exposed to the LA context, spent indeed more time in the center of the open field than vehicle-treated rats exposed to the HA context. This result indicates that the LA environment may induce a lower level of emotional activation (Prut and Belzung, 2003).

The view that LA condition induces a lower level of emotional activation is also supported by behavioral analysis derived from sessions 2 to 6 of the task, in which the rats encountered different objects, also located in different positions in the open-field arena. Rats exposed to the LA context spent more time investigating the objects than rats exposed to the HA context, suggesting that a lower state of anxiety urges animals to better explore the objects (Crawley, 1985). Concerning the cognitive performance, the different level of emotional activation derived by exposure to the two environmental conditions did not influence the cognitive parameters measured in the task. Indeed, vehicle-treated rats exposed to either HA or LA conditions were equally able to recognize the object substitution but failed to respond to the object displacement. Interestingly, Ricceri and co-workers (Ricceri et al., 2000) showed that only 90-day-old mice were able to discriminate a spatial object rearrangement, while 46-day-old mice were not. In our study, we used young adult rats; this leaves open the possibility that the ability to discriminate a spatial change has to be still developed by rats at this age. Moreover, our findings are in accordance with the general assumption that the capability to recognize a new setting of the environment is important for the species survival, but the impact of the object novelty is more salient than a spatial rearrangement with the same objects (Mumby et al., 2002).

Extensive evidence demonstrates that the endocannabinoid system is a crucial regulator of emotionality and cognition (Marsicano et al., 2002; Laviolette and Grace, 2006a,b; Campolongo et al., 2009a,b; Mackowiak et al., 2009; Abush and Akirav, 2010; Akirav, 2011; Trezza et al., 2012). Although the neurobiological mechanisms underlying cannabinoid manipulation of emotional and cognitive functions have not yet been completely elucidated, previous evidence demonstrates that the anxiolytic effects induced by pharmacological enhancement of endocannabinoid tone strongly depend on the emotional state at the time of testing (Patel and Hillard, 2006) and that these effects are modulated by the level of emotional reactivity induced by high or low aversive experimental conditions (Haller et al., 2009).

To further shed light on the role of environmental aversiveness in cannabinoid modulation of emotionality and cognitive performance, we investigated whether exogenous manipulation of the endocannabinoid system influences rat behavior in the Spatial Open Field task in experimental conditions characterized by either a HA or LA state. Our findings clearly show that the effects of the endocannabinoid transport inhibitor AM404 on cognitive responses in the Spatial Open Field test strongly depend on the level of emotionality at the time of testing. Indeed, AM404 administration impaired the rat capability to discriminate between a familiar and a new object only in rats exposed to the HA condition.

Several studies have shown that CB1 receptor agonists produce anxiolytic- (Patel and Hillard, 2006; Scherma et al., 2008) or anxiogenic-like (Viveros et al., 2005; Patel and Hillard, 2006) effects, depending on the dose tested. Conversely, indirect cannabinoid agonists, that increase ongoing endocannabinoid signaling by interfering with their deactivation, induce anxiolytic-like effects without anxiogenic responses also when administered at high doses. For instance, the FAAH inhibitor URB597 produces anxiolytic-like effects in the elevated zero-maze and in the ultrasonic vocalization test in rats (Kathuria et al., 2003). In accordance with these findings, FAAH knockout mice exhibit

an anxiolytic-like phenotype in the elevated plus-maze and in the light-dark box tests (Naidu et al., 2007; Moreira et al., 2008, 2009). Anxiolytic-like effects can also be induced by the inhibition of the endocannabinoid transport operated by endocannabinoid uptake inhibitors like AM404 (Beltramo et al., 1997; Beltramo and Piomelli, 2000). Thus, it has been demonstrated that the systemic administration of AM404 produces anxiolytic-like effects in three rat models of anxiety: elevated plus maze, defensive withdrawal, and separation-induced ultrasonic vocalization tests, and these effects are blocked by the administration of the CB antagonist rimonabant (Bortolato et al., 2006; Patel and Hillard, 2006). Nevertheless, it should be noted that in an another study Moreira and co-workers (Moreira et al., 2007) found that co-administration of anandamide and AM404 in the rat periaqueductal gray (a brain structure related to aversive response) elicited anxiolytic-like responses in the elevated plus maze test, whereas AM404 alone did not. In the present study, we found that administration of AM404 did not influence the emotional parameters taken onto consideration in the Spatial Open Field test, like the time spent in the central part of the arena during the first session of the task. However, it is important to note that, while AM404 administration did not influence the investigation of the objects through session 2–6 in a context characterized by a low-level of emotional activation, rats treated with the lower dose of AM404 and exposed to a stressful environment spent less time investigating objects, whereas the higher doses re-established the investigation activity at similar level of the vehicle-treated rats. The inhibition or the maintenance of the investigative behavior can be related to an anxiogenic or an anxiolytic phenotype, respectively (Crawley, 1985). It is possible to speculate that this biphasic effect may depend on a differential regulation activity on both GABAergic and gutamatergic neurons mediated by different doses of the endocannabinoid transport inhibitor (Foldy et al., 2007; Hashimotodani et al., 2007).

Regarding the cognitive performance, here we show for the first time that a pharmacologically-induced enhancement of endocannabinoid tone differentially modulates memory recognition in rats depending on different emotional states and different nature of the considered cognitive parameters (e.g., either spatial or novel object discrimination).

Concerning the object displacement, although the results did not reach any statistical significance it could be important to note that the treatment effect profile resemble a trend of a typical U-shaped dose response curve, in accordance with other results showing a similar dose-dependent biphasic response induced by cannabinoids, particularly by anandamide (Sulcova et al., 1998) and by the psychoactive constituent of *Cannabis sativa* preparation Δ9-tetrahydrocannabinol (Onaivi et al., 1990; Valjent et al., 2001). Concerning the object substitution, the lower doses of AM404 disrupted the ability to recognize a novel object in a stressful condition (HA) but not in a low arousal context (LA). It is well-established that the capability to recognize a new setting of the environment is important for species survival, but also that the impact of the object novelty is more salient than a spatial rearrangement with the same objects (Mumby et al., 2002). However, the capability to discriminate a novel object in the arena can be lost under particular circumstances such as in a more stressful context, after repeated exposure to an aversive environment and experimental manipulation of the endocannabinoid tone as in the present study (Save et al., 1992; Mumby et al., 2002; Hebda-Bauer et al., 2010).

These data confirm previous findings showing similar effects in humans and laboratory animals where acute or chronic exposure to the psychoactive constituent of cannabis, Δ9-tetrahydrocannabinol, induces impairment in cognitive function (Egerton et al., 2006; Ranganathan and D'Souza, 2006; Solowij and Battisti, 2008; Campolongo et al., 2009c, 2011; D'Souza et al., 2009; Sofuoglu et al., 2010). In rodents, cannabinoid direct agonists induce impairment in several cognitive performances such as spatial learning, working memory, and attentional processes (Presburger and Robinson, 1999; Hampson and Deadwyler, 2000; Verrico et al., 2004; Robinson et al., 2007; Boucher et al., 2009, 2011). It is possible to speculate that these effects derive from cannabinoid-mediated disruption of cortical and hippocampal activity, crucially involved in encoding of the stimulus and making cognitive associations (Robbe et al., 2006; Deadwyler et al., 2007; Robbe and Buzsaki, 2009). The present results confirm the hypothesis that cannabinoid drugs, depending on the dose tested and the emotional state of the subject, could induce different effects on short-term memory parameters. The dissimilar effects induced by exposure to a different emotional state could depend on the activation of the hypothalamic-pituitary-adrenal (HPA) axis triggered by a HA context and to the subsequent release of stress hormones, such as glucocorticoids. It is well-known that this axis plays a crucial role in the stress response and that these hormones differentially modulate cognitive functions (Roozendaal and McGaugh, 1997; Mizoguchi et al., 2004; Atsak et al., 2011). In particular, de Quervain and co-workers (2009) reported that elevated glucocorticoid levels, elicited by aversive contexts, impair memory retrieval, and working memory. Moreover, further studies, conducted by our group, shed light on the crucial role of endocannabinoid signaling in the basolateral complex of the amygdala in modulating consolidation of aversive memory by an interaction with the glucocorticoid system (Campolongo et al., 2009a,b; Hill et al., 2010; Atsak et al., 2011).

Taken together, the present findings support the hypothesis of a fundamental role of the environment in influencing both the behavioral and cognitive outcomes in the Spatial Open Field task. Most importantly, it emerges that drugs that enhance endocannabinoid signaling by interfering with endocannabinoid deactivation induce different effects on short-term memory performance depending on the level of emotional arousal induced by different environmental settings.

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Emotional arousal and multiple memory systems in the mammalian brain

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Emotional arousal induced by stress and/or anxiety can exert complex effects on learning and memory processes in mammals. Recent studies have begun to link study of the influence of emotional arousal on memory with earlier research indicating that memory is organized in multiple systems in the brain that differ in terms of the "type" of memory they mediate. Specifically, these studies have examined whether emotional arousal may have a differential effect on the "cognitive" and stimulus-response "habit" memory processes sub-served by the hippocampus and dorsal striatum, respectively. Evidence indicates that stress or the peripheral injection of anxiogenic drugs can bias animals and humans toward the use of striatal-dependent habit memory in dual-solution tasks in which both hippocampal and striatal-based strategies can provide an adequate solution. A bias toward the use of habit memory can also be produced by intra-basolateral amygdala (BLA) administration of anxiogenic drugs, consistent with the well documented role of efferent projections of this brain region in mediating the modulatory influence of emotional arousal on memory. In some learning situations, the bias toward the use of habit memory produced by emotional arousal appears to result from an impairing effect on hippocampus-dependent cognitive memory. Further research examining the neural mechanisms linking emotion and the relative use of multiple memory systems should prove useful in view of the potential role for maladaptive habitual behaviors in various human psychopathologies.

Keywords: emotion, memory, amygdala, striatum, hippocampus, learning, anxiety, stress

The influence of "emotional arousal" including in particular the effects of stress and anxiety on learning and memory has received extensive investigation (for reviews see Joels et al., 2006; Shors, 2006). Consistent with the hypothesis that emotional arousal can serve to modulate memory processes in a bi-directional manner, both enhancing and impairing effects of stress and anxiety have been observed in lower animal and human studies. In addition, investigation of brain regions that in part mediate the modulatory effects of emotional arousal on memory has focused largely on various limbic system structures, in particular the basolateral amygdala (BLA) (for review see McGaugh, 2004). Studies examining the organization of memory in the mammalian brain indicate the existence of multiple memory systems, raising the question of whether emotional arousal may differentially influence the use of different brain systems in a given learning situation. This hypothesis is examined in the present brief review, in which dissociable roles of the hippocampus and dorsal striatum in "cognitive" and "habit" memory, respectively, are first described. This is followed by a discussion of recent studies in lower animals indicating a differential influence of emotional arousal on the *relative* use of multiple memory systems. Evidence that the BLA mediates the influence of emotional arousal on multiple memory systems in lower animals is presented. Finally, recent work examining the effects of stress on the use of multiple memory systems in humans is described, and the hypothesis that the modulatory influence of emotional arousal on cognitive and habit memory systems may be relevant to understanding various human psychopathologies is briefly considered.

NEUROBIOLOGICAL EVIDENCE FOR MULTIPLE MEMORY SYSTEMS: HIPPOCAMPUS AND DORSAL STRIATUM

Extensive evidence from studies employing a variety of mammalian species indicates that memory is organized in multiple brain systems that differ in terms of the type of memory they mediate (for reviews see Squire et al., 1993; Packard and Knowlton, 2002; White and McDonald, 2002; Squire, 2004). Neurobiological evidence that the brain contains multiple memory systems has led to the proposal of several "dual-memory" theories designed to define the psychological operating principles that distinguish different forms of memory (for review see Kesner, 1998). Each of these theories was derived primarily from a comparison of the pattern of spared and impaired learning and memory functions observed following damage to the hippocampal system. In addition, the operating principles described in many dual-memory theories, particularly those derived from the animal literature, are heavily influenced by the classical debate between "cognitive" (e.g., Tolman, 1932) and "stimulus-response" (e.g., Thorndike, 1933; Hull, 1943) animal learning theorists. For example, the memory functions of the mammalian hippocampal system have been described as essentially neo-Tolmanian in nature, while the types of learning spared following hippocampal system damage are often readily interpreted by Hullian-like

S-R or "habit" learning theories (e.g., Hirsh, 1974; O'Keefe and Nadel, 1978; Mishkin and Petri, 1984).

In rats, evidence for the multiple memory systems hypothesis is found in experiments comparing the effects of manipulations of the hippocampal system and caudate-putamen (i.e., dorsal striatum). In studies using pairs of tasks with similar motivational, sensory, and motoric processes, lesions of the rat hippocampal system and dorsal striatum result in a double dissociation of task acquisition (Packard et al., 1989; Packard and McGaugh, 1992; McDonald and White, 1993; Kesner et al., 1993). For example, lesions of the fimbria-fornix impair acquisition of "win-shift" behavior in the radial maze, a learning task that requires rats to remember which maze arms have been visited within a daily training session (Packard et al., 1989). However, fornix lesions facilitate acquisition of a simultaneous visual discrimination "win-stay" radial maze task, in which food baited maze arms are signaled by a light cue (Packard et al., 1989). In contrast, lesions of the dorsal striatum impair acquisition of the win-stay radial maze task, but have no effect on acquisition of win-shift behavior (Packard et al., 1989; McDonald and White, 1993). Another early experiment in rats demonstrating a double dissociation between the mnemonic functions of the hippocampal system and dorsal striatum used two versions of a two-platform water maze task. In this task, two rubber balls protruding above the water serve as cues. One ball (correct) is on a rectangular platform that can be mounted to escape the water, and the other ball (incorrect) is mounted on a thin rod and thus does not provide escape. The two balls also differ in visual appearance (i.e., vertical versus horizontal black/white stripes). In a cognitive version of the task, the correct platform is located in the same location on every trial, but the visual appearance of the ball varies. Thus, this version of the task requires rats to learn to approach the correct ball on the basis of spatial location, and not visual pattern. In the S-R habit version of the task, the correct platform is located in different spatial locations across trials, but the visual pattern is consistent. Thus, this task can be acquired by learning an approach response to the visual cue (i.e., pattern discrimination). Lesions of the fornix, but not the dorsal striatum, impair acquisition of the cognitive task; whereas lesions of the dorsal striatum, and not hippocampus, impair acquisition of the habit task (Packard and McGaugh, 1992).

A further example of the differential roles of the hippocampus and dorsal striatum in memory involves the use of a plus-maze task that served as a "battleground" for the debate between cognitive and S-R learning theorists during the late 1940's–early 1950's (for review see Restle, 1957). The plus-maze apparatus allows an animal to approach a goal box (e.g., east or west) from one of two start boxes (e.g., north or south). In a "dual-solution" version of the task, rats are trained to start from the same start box (e.g., south) and obtain food in a consistently baited goal box (e.g., west). In describing the manner in which rats acquire this task, cognitive learning theorists argued that rats learn the spatial location of the food reward (i.e., "place" learning). However, stimulus-response learning theorists argued that rats instead learn to make a specific body turn at the choice point (i.e., "response" learning). The use of these two possible learning mechanisms can be assessed in a probe trial administered after training, in which rats are started from the opposite start box (e.g., north). On the probe trial, rats that approach the spatial location that contained food during training are designated "place" learners, whereas rats that make the same body turn that was reinforced during training are designated "response" learners.

Although intact rats can use both types of learning in performing the task (depending in part on intra- and extra-maze environmental conditions; for review see Restle, 1957), the multiple memory systems hypothesis raises the possibility that these two types of learning may have distinct neural substrates. This hypothesis was addressed in a plus-maze study designed to differentiate the role of the hippocampus and the dorsal striatum in memory (Packard and McGaugh, 1996). Rats were trained in a daily session from the same start box to obtain food from a consistently baited goal box. Following a week of daily training the animals were given a probe trial to assess the use of place or response learning. Prior to the probe trial, rats received intra-dorsal striatal or intra-hippocampal injections of either vehicle saline or the local anesthetic lidocaine, a compound that produces a localized and reversible neural inactivation via a blockade of voltage-gated sodium channels. Rats receiving vehicle injections into the hippocampus or dorsal striatum were predominantly place learners on the probe trial. Intra-hippocampal, but not intra-striatal injections of lidocaine blocked expression of place learning. Thus, the functional integrity of the hippocampus, but not dorsal striatum is necessary for expression of place learning.

In order to assess whether the dorsal striatum might be selectively involved in response learning, we took advantage of previous findings indicating that with extended training in the plus-maze intact rats "switch" from the use of place learning to response learning (for review Restle, 1957). Accordingly, we trained the rats for an additional week and then administered a second probe trial. Rats receiving vehicle injections into either the hippocampus or dorsal striatum were predominantly response learners on the second probe trial, confirming previous reports of a shift from the use of place information to response learning with extended training. Intra-hippocampal injections of lidocaine had no effect on the expression of response learning. In contrast, rats receiving intra-striatal lidocaine displayed *place* learning (i.e., a blockade of the use of response learning observed in control rats). These findings provide further evidence for the differential roles of the hippocampus and the dorsal striatum in memory. They also suggest that, in this dual-solution task, place learning is acquired earlier than response learning and that with extended training the control of learned behavior "shifts" from the hippocampus to the dorsal striatum.

Finally, in addition to lesion studies, double dissociations between the roles of the hippocampus and the dorsal striatum in memory have also been observed following post-training intracerebral drug injections (e.g., Packard and White, 1991; Packard et al., 1994; Packard and Teather, 1997, 1998). For example, post-training intra-hippocampal injections of dopaminergic agonists selectively enhance memory in a win-shift radial maze task, while similar injections into the dorsal striatum selectively enhance memory in a win-stay radial maze task (Packard and White, 1991). Moreover, post-training intra-hippocampal injections of the glutamatergic NMDA receptor antagonist AP5 selectively

impair memory in a hidden platform water maze task, while similar injections into the dorsal striatum selectively impair memory in a visible platform water maze task (Packard and Teather, 1997). In both the hippocampus and the dorsal striatum, the effects of the post-training treatments are time-dependent; injections delayed 2 h post-training have no effect on memory. The time-dependent nature of these post-training injections strongly implicates these brain regions in the modulation of memory processes (McGaugh, 1966), and the task-dependent nature of the treatments indicate selective roles for the hippocampus and dorsal striatum in different types of memory.

Finally, when taken together, double dissociations between the mnemonic functions of the hippocampus and the dorsal striatum in cognitive and habit memory have also been demonstrated in other mammalian species, including monkeys (e.g., Teng et al., 2000; Fernandez-Ruiz et al., 2001) and humans (e.g., Martone et al., 1984; Heindel et al., 1988; Butters et al., 1994; Knowlton et al., 1996).

EMOTIONAL AROUSAL AS A FACTOR INFLUENCING THE USE OF MULTIPLE MEMORY SYSTEMS

In view of the numerous studies supporting a multiple systems view of memory organization in the mammalian brain, there has been an increasing interest in examining various factors that might influence the *relative* use of these different systems. In this context we have conducted several experiments assessing the effects of "emotional arousal" produced by acute stress or drug-induced anxiety on the use of cognitive and habit memory systems. For example, in one study (Kim et al., 2001), rats were exposed to a pre-training stress regimen (restraint stress and intermittent tail-shock) and trained in a dual-solution water maze task in which a visible escape platform is always located in the same spatial location. Training in this task appears to involve a parallel activation (McDonald and White, 1994) of hippocampus-dependent cognitive memory (swim to the same spatial location) and dorsal striatal-dependent habit memory (swim to the visible cue). On a probe trial given after task acquisition, the use of these two learning strategies is assessed by moving the visibly cued platform to a new spatial location and observing whether rats continue to swim to the old location or, alternatively, continue to approach the visible cue. Rats that were administered the pre-training stress-regimen acquired the task at a normal rate. However, on a probe trial 24 h after training, the previously stressed rats displayed a predominant use of habit learning, approaching the cued platform in its new location and showing significantly fewer visits to the old spatial location (Kim et al., 2001). These findings suggest that, in a task in which both hippocampus-dependent and dorsal striatal-dependent learning is adequate for acquisition, acute stress may bias rats toward the use of striatal-dependent habit memory.

We have expanded on these findings by examining the effects of drug-induced anxiety on the relative use of multiple memory systems. Accordingly, we selected doses of the α-2 adrenoreceptor antagonists yohimbine and RS 79948–197 that have been shown to possess anxiogenic properties in rats (e.g., Handley and Mithani, 1984; Guy and Gardner, 1985; White and Birkle, 2001). Using a water maze version of the dual-solution plus-maze task described earlier, we observed that pre-training peripheral injections of either drug produced a robust use of response learning relative to place learning (Packard and Wingard, 2004). A similar bias toward the use of response learning in the dual-solution water maze task is also observed if the drugs are injected prior to memory retrieval (i.e., prior to the probe trial), and importantly the effects of pre-training or pre-retrieval injections of RS 79948–197 are not due to state-dependency (Elliot and Packard, 2008). In addition, pre-training exposure to an ecologically valid stressor (trimethlythiazoline, a component of fox feces odor) also biases rats toward the use of dorsal-striatal response learning in the dual-solution water plus-maze task (Packard and Carlin, unpublished data). Taken together, experiments involving the administration of acute stress or anxiogenic drug injections in lower animals suggest that, at least in some learning situations, robust levels of emotional arousal may bias the brain toward the use of a habit memory system. Interestingly, lower levels of trait anxiety have been recently shown to correlate positively with place recognition memory and with a preference for the use of hippocampus-dependent place learning in rats (Hawley et al., 2011).

EMOTIONAL AROUSAL AND MULTIPLE MEMORY SYSTEMS: ROLE OF BASOLATERAL AMYGDALA

The studies reviewed above suggest that acute stress or peripheral anxiogenic drug injections may influence the relative use of multiple memory systems. However, they do not directly identify the neuroanatomical structure(s) that confer the ability of emotional arousal to favor habit learning and memory. In this context we have focused on investigating a potential role for the BLA. There are at least two lines of evidence from animal studies that support the hypothesis that the BLA may mediate a modulatory influence of emotional arousal on different memory systems. First, this brain structure has been historically linked to emotional behavior in mammals (e.g., Kluver and Bucy, 1939), and in rats intra-BLA injection of various drugs induces an anxiogenic behavioral and physiological profile (e.g., Nagy et al., 1979; Scheel-Kruger and Petersen, 1982; Sanders and Shekhar, 1991). Second, decades of research has implicated the BLA as a critical brain site for the memory modulatory effects of drugs that influence several transmitter systems, including those activated by emotional arousal (for review see McGaugh, 2004). According to the hypothesis that the BLA functions as a "general" memory modulatory system, efferent projections of the BLA influence the consolidation of memory in other brain structures. Consistent with this idea, extensive evidence indicates that the BLA modulates memory processes occurring in both the hippocampus and the dorsal striatum (Packard et al., 1994, 1996; Packard and Teather, 1998; Roozendaal and McGaugh, 1996, 1997).

In order to examine whether the bias toward habit memory produced by peripheral anxiogenic drug administration may involve the BLA, rats trained in the dual-solution plus-maze tasks received injections of RS 79948–197 directly into this brain structure. On the later drug-free probe trial, these rats showed a significant use of response learning relative to control rats (Packard and Wingard, 2004). This finding indicates that intra-BLA injections of RS 79948–197 mimic the effects of peripheral

administration of the drug. Moreover, we subsequently demonstrated that the dose of RS 79948–197 that produces this memory modulatory influence is also anxiogenic when injected into the BLA (Wingard and Packard, 2008).

One question raised by these findings in the dual-solution plus-maze task concerns whether intra-BLA injections bias rats toward the use of habit learning by *directly* facilitating striatal-dependent response learning, or in a perhaps more *indirect* manner by impairing hippocampus-dependent place learning. In order to address this question, we trained rats in "single-solution" versions of the water plus-maze task that *required* rats to use either response or place learning. In the single-solution plus-maze tasks, the start points used varied between North and South. In the response task, the spatial location of the escape platform varied equally across trials (East or West) and the same body turn response (e.g., left) was consistently reinforced. In the place task, the escape platform was always located in the same spatial location (e.g., West) and the body turn responses (left and right) were equally reinforced. Separate groups of rats trained in these tasks and receiving post-training intra-BLA injections of an anxiogenic dose of RS 79948–197 displayed *enhanced* acquisition of the response learning task and *impaired* acquisition of the place learning task (Wingard and Packard, 2008). This behavioral profile is consistent with the hypothesis that the facilitation of response learning produced by the drug results from an impairing effect on hippocampus-dependent place learning. Indeed, we have previously observed that post-training neural inactivation of the dorsal hippocampus also enhances response learning and impairs place learning. An interfering competitive action of the hippocampus during training in the single-solution response task presumably occurs because the spatial location of the escape platform varies across trials (Schroeder et al., 2002; Chang and Gold, 2003; for a review on competition between multiple memory systems see Poldrack and Packard, 2003).

In a final recent set of experiments, we examined whether the BLA is critical for the ability of RS 79948–197 to both enhance response learning and impair place learning when the drug is administered peripherally. Separate groups of rats trained in either the single-solution response or place tasks received post-training peripheral injection of an anxiogenic dose of RS 79948–197 and *concurrent* intra-BLA injections of the local anesthetic bupivacaine. In this study, both the enhancing *and* the impairing effect of peripheral administration of RS 79948–197 on response and place learning, respectively, were blocked by neural inactivation of the BLA (Packard and Gabriele, 2009). These findings provide compelling evidence for a critical role for the BLA in mediating the influence of emotional arousal on different types of memory.

In summary, lower animal studies indicate that peripheral or intra-BLA administration of an anxiogenic drug(s) can bias rats toward the use of dorsal-striatal habit memory and that the influence of the BLA on the relative use of multiple memory systems appears to modulate the degree of interference between cognitive and habit memory. In the remaining discussion, we consider the extent to which stress may affect hippocampal and dorsal striatal morphology, whether emotional arousal may also influence the relative use of multiple memory systems in humans, and briefly describe possible implications of this hypothesis for understanding the role of learning and memory processes in various psychopathologies.

THE HIPPOCAMPUS, DORSAL STRIATUM, AND STRESS

When an organism senses a threat, stress hormones are released via the HPA axis and bear a significant and unequal impact upon various brain structures related to learning and memory (for review see McGaugh, 2002). Among these brain structures, the hippocampus and dorsal striatum appear to be differentially affected by stress. In rats, chronic stress causes atrophy of hippocampal neurons of the CA3 region (Watanabe et al., 1992; Magariños and McEwen, 1995) and hypertrophy in the dorsal striatum (Dias-Ferreira et al., 2009). Also, in a longitudinal MRI study, rats subjected to 21 days of chronic restraint stress displayed a 3% reduction in hippocampal gray matter volume, an effect that was not observed in non-stressed controls (Lee et al., 2009). fMRI studies reveal potentially similar results in human subjects with anxiety disorders. Numerous studies have shown that people who have been exposed to trauma and developed post-traumatic stress disorder (PTSD) generally have smaller hippocampi than those who did not develop PTSD (Gilbertson et al., 2002; Bremner et al., 2003; Lindauer et al., 2004; Carrion et al., 2007). In addition, there is evidence that people with PTSD or obsessive-compulsive disorder (OCD) have enlarged caudate nuclei (Looi et al., 2009; Radua et al., 2010). It is unclear whether chronic stress affects the relative size of these brain structures or if smaller hippocampi and larger caudate nuclei precede the development of these anxiety disorders. Indeed, the nature of the relationship between hippocampal gray matter volume and PTSD has attracted considerable debate (Bremner, 2001; Pitman, 2001). In favor of a causal relationship, there is evidence that stressful life events can negatively affect hippocampal morphology in humans. Using a longitudinal MRI paradigm, researchers measured hippocampal gray matter volume at two time points, separated by a three month interval. The number of stressful life events experienced during the three-month interval was positively correlated with a reduction in gray matter volume of the right hippocampus (Papagni et al., 2011), suggesting that the relationship between PTSD and hippocampal volume may be causal.

Interestingly, a single stressful experience may be sufficient to affect the morphology of the hippocampal formation. Several studies have shown that acute stress suppresses neurogenesis of progenitor cells in the dentate gyrus of rodents (Galea et al., 1996; Gould et al., 1997; Tanaput et al., 2001) and non-human primates (Gould et al., 1998). Moreover, in humans hydrocortisone administration decreased activation of the hippocampus in the retrieval phase of a declarative memory task (Oei et al., 2007).

It is less clear whether acute or chronic stress affects the function of the dorsal striatum. However, evidence from human neuroimaging studies suggests that the dorsal striatum may play a role in the processing of negative stimuli. In healthy subjects, the dorsal striatum responds intensely when viewing unpleasant pictures, relative to positive or neutral pictures (Carretie et al., 2009). In addition, in anxious subjects, dorsal striatal activation increases when reading negative words as opposed to positive or neutral ones (Roiser et al., 2008). Lastly, subjects with Huntington's

disease (a disease associated with deterioration of the dorsal striatum) are impaired in their ability to recognize negative facial expressions (Gray et al., 1997; Johnson et al., 2007).

THE AFFECT OF STRESS ON THE RELATIVE USE OF MULTIPLE MEMORY SYSTEMS IN HUMANS

As described earlier, numerous studies in rats suggest a dynamic impact of emotional arousal on hippocampus-dependent and dorsal striatal-dependent memory systems. Specifically, acute stress and/or anxiety appears to bias rats to solve dual solution tasks by employing an S-R habit learning strategy, at the expense of the competing cognitive memory system (Packard and Wingard, 2004; Wingard and Packard, 2008). To the extent that the influence of emotional arousal on the hippocampus and dorsal striatum is similar in rodents and humans, it is possible that the effect of stress on the *relative* use of memory systems observed in rodents may also be observed in humans.

Indeed, recent studies building on the earlier research in lower animals indicate that both acute and chronic stress may also bias human subjects to implement an S-R habit learning strategy to solve a dual solution task. For example, Schwabe et al. (2007) trained subjects to locate a "win-card" in a 3D model of a room. Over 12 trials, subjects could locate the win-card by using a spatial strategy (i.e., the card is always in the same spatial location) or a stimulus-response strategy (i.e., the card is always next to the plant). On the 13th trial, the plant was moved to a different location which allowed the experimenters to assess the type of learning strategy used. Prior to training, subjects were exposed to an acute stressor consisting of giving a speech and performing mental math in front of an audience. Subjects in this stressed condition implemented an S-R strategy to locate the win-card significantly more often than controls. Interestingly, high salivary cortisol at the time of learning predicted habit behavior in stressed and non-stressed conditions. In a subsequent study, subjects with higher scores on a chronic stress questionnaire implemented an S-R strategy in a 2-D dual solution task significantly more often than subjects with lower scores (Schwabe et al., 2008a). Therefore, similar to the effects of acute stress, chronic stress also appears to favor habit learning, at the expense of spatial learning, in humans.

Consistent with these findings, another study assessed the effect of emotional arousal on retention of the striatal-dependent weather prediction task (Steidl et al., 2011). For each trial in this task, subjects are asked to predict the weather based on a random set of cards depicting different abstract shapes. Each possible combination of cards has a predetermined probability of signifying rain or sunshine, and after each prediction subjects are given feedback as to whether their prediction was correct or incorrect. In this particular study, in order to manipulate the level of emotional arousal, subjects were presented with either "arousing" or "neutral" pictures during acquisition of the task. In a retention test given 1.5 months after training, subjects who had viewed the arousing pictures exhibited sustained memory for the task, whereas subjects who had viewed the neutral pictures exhibited considerable memory decay. Previous studies using fMRI or enlisting subjects with selective damage to the basal ganglia have indicated that the striatum has a central role in the initial acquisition of the weather prediction task (Knowlton et al., 1996; Poldrack et al., 1999). Therefore, it could be interpreted that heightened emotional arousal may have further enhanced the role of the striatum during training, thus strengthening the memory and improving performance in the retention test. However, it should be noted that the hippocampus is implicated in later performance of the weather prediction task (Knowlton et al., 1994, 1996), suggesting that a hippocampus-dependent learning system may have also had a role in the enhanced retention (Steidl et al., 2006).

Interestingly, some research has shown that stress promotes habit behavior in instrumental learning tasks as well. In one study (Schwabe and Wolf, 2009), human subjects were exposed to the socially-evaluated cold pressor test or a non-stressed control condition and then trained on two instrumental tasks, each associated with a distinct food outcome. After training, one of the food outcomes was devalued by feeding the subject with the food until satiation. In a subsequent extinction test, subjects exposed to pre-training stress continued performing the same instrumental response despite it being associated with the devalued food outcome (i.e., pre-training stress prompted habitual behavior). Subjects unexposed to stress decreased the instrumental behavior associated with the devalued food outcome, suggesting the use of a more cognitive, goal-directed learning system. In a separate fMRI study, it was observed that habit behavior in the food devaluation paradigm was associated with increased activation of the dorsal striatum, while goal-directed behavior was associated with increased activation of the ventromedial prefrontal cortex (Tricomi et al., 2009). Therefore, stress-induced habit behavior in this task may also represent a shift to a dorsal striatal-dominant activation pattern.

While behavioral stressors appear to bias humans toward the use of a habit memory system at the expense of the competing cognitive system, studies investigating the role of the human stress hormone cortisol seem to yield opposite results. For example, one study observed that low basal cortisol levels were associated with the use of an S-R habit strategy, whereas higher levels were associated with the use of a spatial strategy in a dual solution virtual maze task (Bohbot et al., 2011). However, subjects' cortisol levels did not correlate with their scores on the Perceived Stress Questionnaire, suggesting that sample cortisol levels in this study may not have reflected actual feelings of stress. The effect of stress on the hippocampus is typically described as following an inverted U-shape, with high and low levels of stress leading to impairments and a "middle" level being optimal for hippocampus function. Considering that no subjects reported high stress, the authors suggest that the higher cortisol readings in this study may actually represent the middle of this inverted-U, thus favoring the use of a hippocampus-dependent memory system. Another study investigating the effect of cortisol showed that orally administered hydrocortisone biased women to solve a dual solution task using a spatial strategy, at the expense of the habit system (Schwabe et al., 2008b). Interestingly, exogenous cortisol treatment was also associated with poorer performance in both spatial and response learners. To explain these results in the context of earlier work, the authors hypothesize that under low cortisol levels the hippocampus controls behavioral output. Under moderate levels, hippocampus function declines and the dorsal striatum

seizes control, and under high levels hippocampus and dorsal striatum function decline, but the balance between systems is restored and the hippocampus regains control. It is interesting to note a contrast between this explanation and the one proposed by Bohbot et al. (2011). Whereas Schwabe et al. (2008b) suggest that the hippocampus gains control at low and high cortisol levels, Bohbot et al. (2011) propose that the hippocampus controls behavioral output at medium levels. The different methods used in these studies (e.g., behavioral tasks; monitoring endogenous cortisol vs. manipulating exogenous cortisol) may in part account for this discrepancy. In this context, it is worth noting that human fMRI studies have established a negative correlation between cortisol levels and hippocampus activity (Oei et al., 2007).

Aside from a potential U-effect, there may be another explanation for the seemingly opposite effects of cortisol. Using the instrumental food-devaluation paradigm discussed earlier, researchers found that only concurrent administration of the α_2-noradrenergic receptor antagonist yohimbine and hydrocortisone promoted habit behavior in humans, whereas administration of hydrocortisone alone resulted in goal-directed, cognitive behavior (Schwabe et al., 2010). Therefore, it appears that increases in both cortisol and norepinephrine may be required to induce a habit bias in dual solution tasks. This finding may explain why a behavioral stressor like the socially-evaluated cold pressor test, which increases plasma norepinephrine (Blandini et al., 1992) and salivary cortisol (Schwabe et al., 2008), can induce a habit bias (Schwabe and Wolf, 2009), whereas a pharmacological increase in cortisol alone does not (Schwabe et al., 2008b).

In summary, both chronic and acute stress appears to bias humans to solve dual solution tasks with a habit memory system, consistent with the research in lower animals. However, when manipulating and monitoring cortisol levels, a more complex picture emerges, and the effect of stress on the relative use of memory systems may follow a U-shaped curve or depend on an interaction between both cortisol and norepinephrine.

POTENTIAL MECHANISMS UNDERLYING THE STRESS-MEDIATED HABIT BIAS IN HUMANS

Extensive research indicates that in some learning situations the hippocampus and dorsal striatum vie for control of behavioral output (for review see Poldrack and Packard, 2003). For example, in rats, lesions, or neural inactivation of the hippocampus can lead to enhanced acquisition of striatal-dependent habit tasks (e.g., Packard et al., 1989; Packard and McGaugh, 1992; Schroeder et al., 2002). In view of evidence that high levels of stress can impair hippocampus-dependent learning in rats, it has been suggested that the stress or anxiety-induced shift to habit learning may result from the hippocampus relinquishing control and releasing the habit memory system from competition (Wingard and Packard, 2008). Consistent with this idea, numerous human studies have shown that high levels of stress at the time of encoding or retrieval impair performance in hippocampus-dependent memory tasks (Schwabe et al., 2009; Merz et al., 2010; Schwabe and Wolf, 2010; Thomas et al., 2010). Therefore, in dual solution tasks, it is possible that stressed individuals are more proficient in solving the task with their unimpaired habit system and thus, in order to preserve performance levels, implement an S-R strategy as opposed to a cognitive strategy. Consistent with this interpretation, subjects with lower scores in episodic memory tasks were more likely to be response learners in a dual solution virtual maze task (Bohbot et al., 2011). It is important to note that stressed human subjects may be generally unaware of alternative strategy options (e.g., spatial) for solving a given dual solution task (Schwabe et al., 2007; Schwabe and Wolf, 2009). Therefore, it is unlikely that stressed individuals make a *conscious* decision to abstain from cognitive solutions and opt for a habit learning strategy. Rather, stressed human subjects may rely on their habit system, simply because the available cognitive solutions go unnoticed.

As mentioned earlier, stressful life events are associated with reduced gray matter volume of the right hippocampus in humans (Papagni et al., 2011). This finding may be relevant for understanding the effect of stress on memory systems, particularly as the relative size of the hippocampus and dorsal striatum may predict the learning strategy used. In one study (Bohbot et al., 2007), researchers utilized a virtual eight-arm radial maze that could be solved by associating distal cues (e.g., mountains, trees, etc.) with the location of the correct maze arms (i.e., a spatial strategy) or by memorizing the sequence of left and right arrow presses on the keyboard that lead to the correct maze arms (i.e., an S-R strategy). To determine which strategy a subject used, the distal cues were blocked in the last trial of the experiment. MRI scans revealed that greater density in the hippocampus was positively correlated with the number of errors in the final probe trial (suggesting the use of a spatial strategy) and that greater density in the dorsal striatum was negatively correlated with the number of errors in the probe trial (suggesting the use of an S-R strategy). Chronic stress may potentially exert its influence on multiple memory systems by affecting the relative volume of the hippocampus and dorsal striatum. Whether the more modest morphological changes induced by acute stress could underlie the habit bias remains to be determined.

As previously described, several studies in lower animals indicate that the effects of emotional arousal on memory depend on the integrity of the BLA (for review see McGaugh, 2004) and also implicate this brain region in orchestrating the use of multiple memory systems during periods of high emotional arousal (e.g., Packard and Wingard, 2004; Wingard and Packard, 2008; Packard and Gabriele, 2009). Interestingly, human fMRI studies reveal that the degree of amygdala activation during encoding positively correlates with the recall of emotion-laden memories (Hamann et al., 1999; Canli et al., 2000). However, to our knowledge, no studies have investigated the relationship between amygdala activation and the *relative use* of memory systems in humans.

Finally, human case studies reveal that acute or chronic anxiety may underlie the development and persistence of several psychopathologies with "habit-like" behavioral features, including for example OCD, post-traumatic stress disorder, and drug addiction. For instance, in OCD, the exaggerated fear of germs or infection may cause a person to "solve the problem" habitually, thus leading to excessive hand washing (Jones and Menzies, 1998). In post-traumatic stress, a significantly traumatic experience can lead to the development of non-context-specific cued recall of the

memory. In this way, some aspects of PTSD may be analogous to the previously described studies showing a stress-induced facilitation of S-R habit (or, cued) learning and concomitant disregard for the spatial context of the learning environment (Schwabe et al., 2007, 2009). In addition, several studies have evidenced a relationship between acute stressors and relapse into habit-like drug seeking behavior in lower animals (Shaham and Stewart, 1995; Shepard et al., 2004; Buffalari and See, 2009) and humans (Kosten et al., 1986; Sinha et al., 1999, 2009). Moreover, there is increasing evidence that the dorsal striatum plays an important role in the expression of drug-seeking behaviors in animals (Ito et al., 2002; Fuchs et al., 2006; See et al., 2007) and humans (Garavan et al., 2000; Volkow et al., 2006). Thus, one might speculate that anxiety and/or stress may influence relapse in drug addiction by favoring the use of habit memory to guide the expression of maladaptive behaviors. Further research examining the relationship between the neural bases of emotional arousal and the relative use of multiple memory systems may prove useful for understanding various human psychopathologies.

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Memory-enhancing intra-basolateral amygdala infusions of clenbuterol increase Arc and CaMKIIα protein expression in the rostral anterior cingulate cortex

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Activation of β-adrenoceptors in the basolateral complex of the amygdala (BLA) modulates memory through interactions with multiple memory systems. The cellular mechanisms for this interaction remain unresolved. Memory-modulating BLA manipulations influence expression of the protein product of the immediate early gene activity-regulated cytoskeletal-associated protein (Arc) in the dorsal hippocampus, and hippocampal expression of Arc protein is critically involved in memory consolidation and long-term potentiation. The present studies examined whether this influence of the BLA is specific to the hippocampus and to Arc protein. Like the hippocampus, the rostral portion of the anterior cingulate cortex (rACC) is involved in the consolidation of inhibitory avoidance (IA) memory, and IA training increases Arc protein in the rACC. Because the BLA interacts with the rACC in the consolidation of IA memory, the rACC is a potential candidate for further studies of BLA modulation of synaptic plasticity. The alpha isoform of the Calcium/Calmodulin-dependent protein kinase II (CaMKIIα) and the immediate early gene c-Fos are involved in long-term potentiation and memory. Both Arc and CaMKIIα proteins can be translated in isolated synapses, where the mRNA is localized, but c-Fos protein remains in the soma. To examine the influence of memory-modulating manipulations of the BLA on expression of these memory and plasticity-associated proteins in the rACC, male Sprague–Dawley rats were trained on an IA task and given intra-BLA infusions of either clenbuterol or lidocaine immediately after training. Findings suggest that noradrenergic stimulation of the BLA may modulate memory consolidation through effects on both synaptic proteins Arc and CaMKIIα, but not the somatic protein c-Fos. Furthermore, protein changes observed in the rACC following BLA manipulations suggest that the influence of the BLA on synaptic proteins is not limited to those in the dorsal hippocampus.

Keywords: synaptic plasticity, stress, emotion, memory consolidation, immediate early gene, local translation, norepinephrine, noradrenalin

INTRODUCTION

Stressful or emotionally arousing events are typically remembered better than emotionally neutral events. Stress hormones, released by the adrenal glands into the bloodstream, assist in preparing an animal to fight or flee by increasing energy resources and promoting attention and vigilance. Extensive evidence indicates that this sympathetic response contributes to the enhancement of memory consolidation through actions on β-adrenoceptors in the basolateral complex of the amygdala (BLA) (Liang et al., 1986; Quirarte et al., 1997; McIntyre et al., 2002; McReynolds et al., 2010). A single footshock is sufficient to produce a long-term contextual memory in rats and increases norepinephrine (NE) levels in the amygdala (Quirarte et al., 1998; McIntyre et al., 2002). Administration of an antagonist to β-adrenoceptors in the BLA blocks the memory enhancement produced by systemic administration of epinephrine (Liang et al., 1986) or the glucocorticoid corticosterone (Quirarte et al., 1997; McReynolds et al., 2010). Further, direct activation of the noradrenergic system in the amygdala, through infusions of NE or the β-adrenoceptor agonist clenbuterol enhance memory of inhibitory avoidance (IA) training, passive avoidance tasks, contextual fear conditioning, conditioned taste aversion, and object recognition training (Gold and van Buskirk, 1975; Gallagher et al., 1977; Ferry and McGaugh, 1999; Hatfield and McGaugh, 1999; LaLumiere et al., 2003; Miranda et al., 2003; McIntyre et al., 2005; Roozendaal et al., 2008).

The amygdala appears to interact with many other brain areas in order to influence these various types of memories (McGaugh, 2004). In fact, memory processing involving the hippocampus (Packard et al., 1994; Malin and McGaugh, 2006), caudate nucleus (Packard et al., 1994), insular cortex (Miranda and McGaugh, 2004), entorhinal cortex (Roesler et al., 2002), medial prefrontal cortex (mPFC) (Roozendaal et al., 2009) and the anterior cingulate cortex (Malin and McGaugh, 2006; Malin et al., 2007) is modulated by manipulations of the amygdala. These findings suggest that the BLA may naturally exercise control over synaptic

plasticity in other regions of the brain that are engaged in memory consolidation.

Expression of the activity-regulated cytoskeletal-associated protein (*Arc*) immediate early gene can be used as a marker for neuronal plasticity based on its rapid appearance and degradation, and the critical involvement of Arc protein in long-term synaptic plasticity and memory (Bramham et al., 2008). Pre-training intra-hippocampal infusions of antisense oligodeoxynucleotides (ODNs), which disrupt translation of Arc protein, impair long-term memory of spatial water maze, and IA training (Guzowski et al., 2000; McIntyre et al., 2005). Infusions of the β-adrenoceptor agonist clenbuterol into the BLA immediately after training on a single-trial IA task enhance both memory retention and expression of Arc protein in the dorsal hippocampus (McIntyre et al., 2005). Intra-BLA infusions of the sodium channel blocker lidocaine into the BLA immediately after training impair memory for IA and decrease Arc protein expression in the dorsal hippocampus. These findings support the hypothesis that the BLA modulates the consolidation of long-term memory through actions on efferent brain regions such as the hippocampus.

Arc is also expressed in areas of the brain outside of the hippocampus. Both aversive and non-aversive tasks increase Arc mRNA in cortical and limbic areas of the brain (Kelly and Deadwyler, 2002, 2003; Ons et al., 2004; Ploski et al., 2008). In mice, fear conditioning increases Arc mRNA in the hippocampus as well as the anterior cingulate cortex, the olfactory bulb, the pyriform, parietal, sensory and motor cortices, and the amygdala (Montag-Sallaz and Montag, 2003). Similarly, stress and fear conditioning increase Arc protein expression in many of the same areas of the rat brain, as well as the mPFC (Kelly and Deadwyler, 2003; Koya et al., 2005; Mikkelsen and Larsen, 2006; Ploski et al., 2008). However, it is unknown whether the BLA can influence these training-induced changes in Arc expression in areas outside of the hippocampus.

The immediate early gene Arc has received attention due to the presence of mRNA in dendritic spines, where it can be translated to protein by interacting with local ribosomes (Steward and Worley, 2002; Yin et al., 2002; Moga et al., 2004; Bramham et al., 2008). Based on evidence that Arc protein is involved in synaptic plasticity, this expression pattern presents a possible mechanism for synapse-specific modifications. However, Arc is not the only known memory-related immediate early gene, or locally translated protein. Our previous findings suggest that the BLA does not modulate expression of the protein product of the immediate early gene c-Fos in the dorsal hippocampus (McIntyre et al., 2005). Both c-Fos protein and mRNA are localized to the soma. Therefore, it is possible that the BLA influences memory through synapse-specific effects by modulating local translation of synaptically localized mRNAs. Another locally translated protein is the alpha-isoform of Calcium/Calmodulin-dependent protein kinase II (CaMKIIα), which appears to play a similar role in memory (Silva et al., 1992a,b). If the BLA modulates memory through an influence specifically on local translation of synaptic proteins, then memory-modulating stimulation of the BLA should produce changes in Arc and CaMKIIα, but not c-Fos protein.

Like the hippocampus, the rostral portion of the anterior cingulate cortex (rACC) appears to interact with the amygdala to consolidate memory of IA training (Malin and McGaugh, 2006; Malin et al., 2007). Activating the rACC with the muscarinic agonist oxotremorine immediately after IA training enhances memory for the nociceptive component of the task, as evidenced by longer retention latencies when tested 48 h later (Malin and McGaugh, 2006). When the BLA is lesioned prior to activation of the rACC by oxotremorine, the memory-enhancing effect is attenuated, showing an interaction between the rACC and the BLA for long-term consolidation of memory (Malin et al., 2007). Indeed, there is substantial evidence showing direct anatomical connections between the ACC and the BLA (Sarter and Markowitsch, 1983; Sripanidkulchai et al., 1984; McDonald, 1991). We recently reported that Arc protein in synapses of the rACC is increased following IA training and rACC expression of Arc protein is necessary for optimal long-term memory for the task (Holloway and McIntyre, 2011). Using western immunoblotting, we found a significant increase in Arc protein expression in tissue taken from the rACC of rats that were trained on a single-trial IA task, compared to the rACC of naive rats. Infusions of antisense ODNs into the rACC immediately or 6 h after IA training disrupted memory for the task (Holloway and McIntyre, 2011). These results provide a basis for exploring the effect of post-training stimulation of BLA β-adrenoceptors on expression of long-term memory and synaptic plasticity-associated proteins Arc, CamKIIα, and c-Fos in the rACC.

METHODS

SUBJECTS

One hundred and fifty four male Sprague-Dawley rats (250–275 g upon arrival) were purchased from Charles River Laboratories (Wilmington, MA). All animals were housed separately in a temperature controlled setting (19.5°C) maintained on a 12 h light/dark cycle (lights on at 7:00 AM), given food and water *ad libitum*. All procedures were conducted in accordance with National Institutes of Health guidelines and approved by The University of Texas at Dallas Institutional Animal Care and Use Committee.

SURGERIES

Rats were anesthetized with isoflurane (1% in O_2, Western Medical Supply). A continuous flow of isoflurane was delivered to the animal throughout the surgery. Once anesthetized, rats' heads were leveled in a stereotaxic device (Stoelting Inc, Wood Dale, IL). Rats were given subcutaneous injections of 0.5 mL marcaine/lidocaine at the incision site. Fifteen millimeter cannulae were placed directly above the BLA [Coordinates in mm: anteroposterior (AP), −2.7 from bregma; mediolateral (ML) ± 5.2 from the midline; dorsoventral (DV) −6.4 from the top of the skull; incisor bar, −3.3 mm from interaural line (Paxinos and Watson, 2005)], mounted with dental cement and secured with skull screws. Stylets (15 mm long insect dissection pins) were inserted into each cannula to maintain patency. Animals were given 3–5 mL of saline subcutaneously to help prevent dehydration, and moved to a warm recovery chamber until they were awake and moving. They were then allowed to recover for one week before training.

INHIBITORY AVOIDANCE (IA) TRAINING

All rats were handled for 2 min/day for five days prior to being trained on an IA task. The IA apparatus was a trough-shaped box, 90 cm in length, with a sliding guillotine style door dividing two compartments. The box was placed in a dark room, and the rats were brought into the room immediately before training. The lighted compartment was 30 cm long, with white plastic sides. A table lamp sat directly over this side, shining down into the compartment. The dark compartment was 60 cm long, with metal sides. Each rat was removed from its home cage and placed in the light compartment facing away from the dark compartment. Once the rat turned around (180°) and crossed over into the dark compartment, the sliding guillotine door was closed and the rat was trapped in the dark compartment. When the rat walked all the way to the end of the box and turned around again, an inescapable 1.0 s shock (0.38 mA for clenbuterol-infused rats; 0.48 mA for lidocaine-infused rats) was applied to the floor plates. The rat remained in the dark compartment for 10 s following the shock before being removed from the IA box and given immediate, post-training intra-BLA infusions of either clenbuterol or lidocaine. Rats were returned to the IA apparatus for memory retention testing 48 h after completion of training. They were again placed in the light compartment. Latency to cross into the dark compartment was recorded and used as a measure of memory. If a rat failed to cross into the dark compartment before ten minutes, it was removed and a time of 600 s was recorded.

INFUSIONS

The β-adrenoceptor agonist clenbuterol (4 ng/0.2 μL dissolved in a vehicle of 0.9% saline; Sigma-Aldrich), the local anesthetic lidocaine (2.0% in saline; Hospira Inc., Lake Forest, IL), or vehicle were administered immediately post-training, through 30 gauge dental needles-extending 2 mm beyond the cannulae-attached to 10 μL Hamilton microsyringes. A volume of 0.2 μL of drug or vehicle was administered over 32 s, at a constant rate, by a KD Scientific (Harvard Instruments) infusion pump. Infusion needles remained in place for an additional 30 s to allow for diffusion of the drug. The doses of clenbuterol and lidocaine were used based on results of previous research showing their effectiveness at enhancing or impairing memory for an IA task (Coleman-Mesches and McGaugh, 1995; McIntyre et al., 2005). For the behavioral task, animals received bilateral post-training intra-BLA infusions. For analysis of protein expression in the ACC, rats were given infusions of clenbuterol or lidocaine into the right or left BLA (and vehicle into the other hemisphere), or vehicle bilaterally (**Figures 3–5**). In this way, each rat served as its own control (as reported previously in McIntyre et al., 2005).

TISSUE PREPARATION

Animals were deeply anesthetized using isoflurane (Western Medical Supply) and brains were rapidly removed and flash frozen by submersion in 2-methylbutane in a dry ice/ethanol bath. Trained animals were euthanized 1 h after training. This time point was chosen based on previous research showing increases in Arc, c-Fos, and CaMKIIα protein in the hippocampus or cortex at this time point (Kleim et al., 1996; Ouyang et al., 1999; Otmakhov et al., 2004; Aslam et al., 2009; Holloway and McIntyre, 2011). The brains were cut coronally just in front of the BLA (−1.20 mm from Bregma) and the anterior portions were saved for tissue collection from the ACC. Only brains with correct cannula placement were used. To determine cannula placement, 40 μm sections were taken from the posterior section with a cryostat, mounted on gelatin subbed slides, and stained with thionin. These slides were analyzed under a light microscope to determine the location of the cannulae and drug infusion sites; **Figure 1** shows a representative photomicrograph indicating a cannula track and drug infusion site in the BLA. Any brains that did not have needle tips in the BLA were not used for data analysis. A series of 500 μm cryosections were collected starting +4.2 mm from bregma and continuing to +2.1 mm from bregma. The ACC was dissected out using a tissue punch kit (0.5 mm diameter), 0.5 mm from midline to the medial edge of each hemisphere and

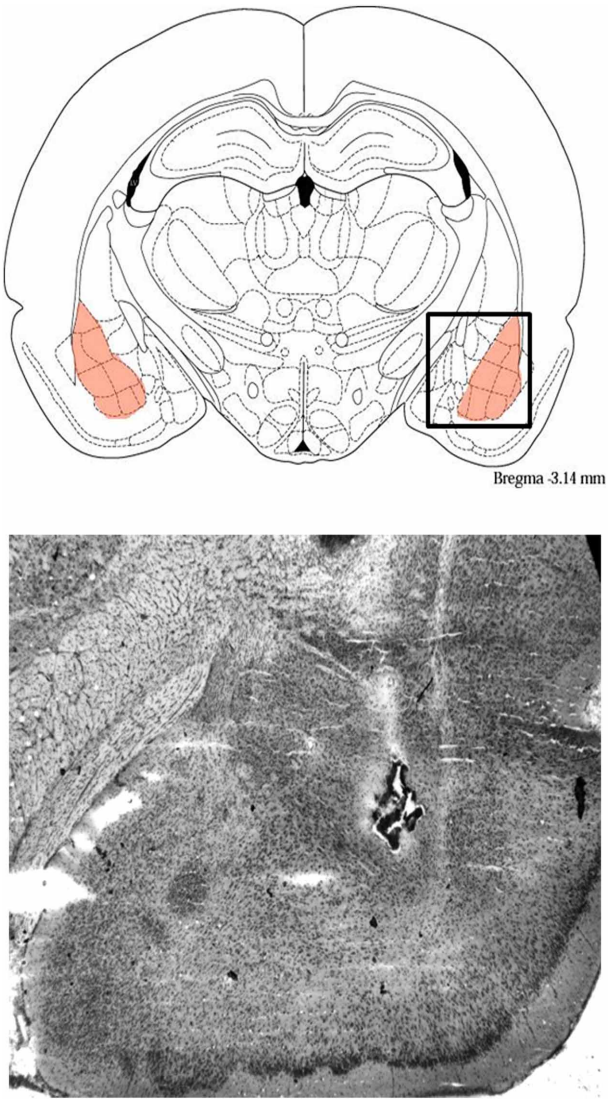

FIGURE 1 | Representative photomicrograph showing cannula tracks and drug infusion site in the BLA, and schematic showing the BLA and surrounding areas.

starting from the top of the brain and continuing –2 mm. The tissue punches were stored at –80°C for later western blot analysis.

WESTERN IMMUNOBLOTTING

Tissue was sonicated in a 0.1 M phosphate buffer, pH 7.4 containing 10% glycerol, 20% protease inhibitor cocktail (Sigma-Aldrich), and 10% phosphatase inhibitor (Sigma-Aldrich). Protein amounts were determined using a Qubit fluorometer and Qubit protein assay kit (Invitrogen). Approximately 15 μg of protein from each sample was heated with a sample buffer and reducing agent (Invitrogen), loaded and then run on 4–12% Bis-Tris MIDI Gels (Invitrogen) using an electrophoresis apparatus. Each gel contained samples from each experimental condition. Gels were transferred by electroblotting onto a nitrocellulose membrane using an iBlot dry-blotting system (Invitrogen). The membrane was then washed in Tris-buffered saline (TBS: 150 mM NaCl/100 mM tris base, pH 7.5) and incubated with primary antibodies diluted in blocking solution (5% non-fat dry milk in TBS-Tween) overnight at 4°C. The primary antibodies were Anti-Arc (rabbit polyclonal; 1:2000, Synaptic Systems), Anti-CaMKIIα (rabbit polyclonal; 1:1000, Cell Signaling), Anti-c-Fos (rabbit polyclonal; 1:250, Santa Cruz Biotechnology) and Anti-Actin (rabbit; 1:1500, Sigma-Aldrich). Two days later the membranes were washed in TBS-Tween, and incubated at room temperature for 1 h in a secondary HRP-linked antibody (goat anti-rabbit; 1:6000, Cell Signaling). Immunoreactivity was detected using chemiluminescence (ECL Western Blot Kit; Pierce). A marker (Invitrogen) was run to determine the relative mobility of immunoreactive bands. For densitometric quantification, films were scanned and converted into TIF files to be analyzed using Image-J software (NIH).

STATISTICAL ANALYSIS

Two-sample *t*-tests were used to analyze IA retention latencies in experiment one. Pair-wise comparisons were made between clenbuterol- or lidocaine-treated animals and vehicle-treated rats. For western blot densitometry, results are expressed as a ratio of Arc, CaMKIIα, and c-Fos to actin, and then expressed as a ratio of drug-infused to vehicle-infused hemisphere, or randomized as a ratio of one hemisphere to the other hemisphere in rats given bilateral vehicle infusions. These values are presented as percent of cage control values to account for film variation. For experiments comparing trained animals to untrained animals, the values are expressed as a percent of experimental/control vehicle group. The final values were analyzed using a Student's *t*-test to make pair-wise comparisons between the groups. A probability level of $p < 0.05$ was considered significant. Data are presented as means ± SEM.

RESULTS

POST-TRAINING INTRA-BLA INFUSIONS OF CLENBUTEROL OR LIDOCAINE SIGNIFICANTLY ENHANCE OR IMPAIR INHIBITORY AVOIDANCE MEMORY

In order to investigate the effects of BLA manipulation on memory, rats were trained on the IA task and received immediate bilateral intra-BLA infusions of clenbuterol ($n = 7$) or vehicle ($n = 6$). Memory retention was tested 48 h later.

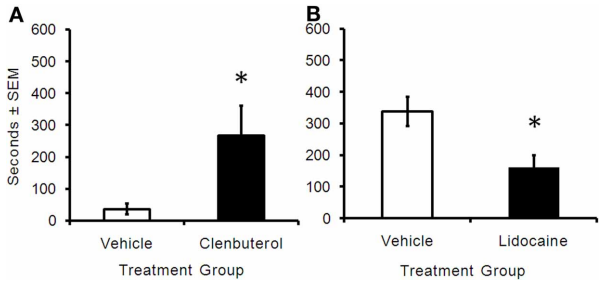

FIGURE 2 | (A) Retention latency times (in seconds) of animals trained on a one-trial inhibitory avoidance task and immediately given intra-BLA infusions of clenbuterol or vehicle. Rats that received infusions of clenbuterol ($m = 267.43 \pm 93.36$, $n = 7$) had a significantly greater cross-over latency 48 h later when compared to animals receiving vehicle infusions ($m = 36 \pm 16.96$, $n = 6$). *$p < 0.05$. **(B)** Rats given post-training intra-BLA infusions of lidocaine ($m = 162 \pm 36.91$, $n = 13$) had significantly lower cross-over latencies than animals given vehicle infusions ($m = 338.69 \pm 46.04$, $n = 13$). *$p < 0.05$.

Clenbuterol-treated rats showed significantly higher retention latencies (mean = 267.42 s) than vehicle-treated rats (mean = 36.00 s; **Figure 2A**; $t(6) = -2.44$, $p < 0.05$), suggesting that clenbuterol-infused animals had an enhanced memory for the task ($p < 0.05$). In another experiment, rats were trained on the IA task and received immediate intra-BLA infusions of lidocaine ($n = 13$) or vehicle ($n = 13$). Rats given intra-BLA infusions of lidocaine showed significantly lower retention latencies (mean = 162.00 s) than rats given intra-BLA infusions of vehicle (mean = 338.70 s; **Figure 2B**; $t(23) = 2.99$, $p < 0.05$) indicating that lidocaine treatment was memory impairing.

ARC AND CAMKIIα PROTEIN LEVELS ARE MODULATED BY INTRA-BLA CLENBUTEROL OR LIDOCAINE INFUSIONS

To examine the interaction between the BLA and the rACC, separate groups of rats were trained on an IA task and given unilateral infusions of clenbuterol or lidocaine into one hemisphere and vehicle into the other hemisphere, or bilateral intra-BLA vehicle infusions. Arc protein expression in the rACC was measured and reported as a ratio of the drug infused hemisphere/vehicle infused hemisphere. Arc protein expression was significantly increased in rACC tissue taken from the clenbuterol infused rats ($n = 10$) when compared to rats ($n = 8$) given bilateral intra-BLA vehicle infusions (**Figure 3A**; $t(15) = -3.72$, $p < 0.05$). Rats given unilateral infusions of lidocaine ($n = 5$) into the BLA showed decreased Arc protein expression in homogenate tissue collected from the rACC compared to the rats ($n = 5$) given bilateral vehicle infusions (**Figure 3B**; $t(8) = 2.96$, $p < 0.05$).

Post-training infusions of the memory-enhancing dose of clenbuterol in the BLA also increased CaMKIIα protein expression in the rACC (**Figure 4A**; $t(9) = -1.94$; $p < 0.05$). Expression of CaMKIIα protein was significantly greater in rats given post-training infusions of clenbuterol ($n = 5$) than vehicle-treated controls ($n = 6$). Likewise, CaMKIIα protein expression in tissue collected from the rACC was significantly lower in rats given intra-BLA lidocaine infusions ($n = 7$) than vehicle-treated controls ($n = 7$) (**Figure 4B**; $t(12) = 3.18$; $p < 0.05$).

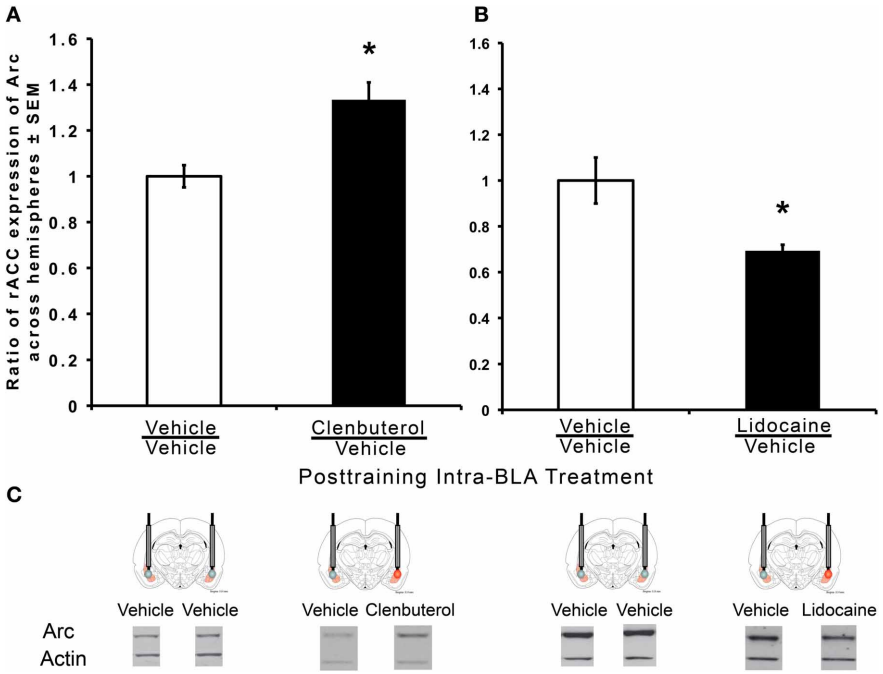

FIGURE 3 | Memory-enhancing intra-BLA infusions of clenbuterol increase Arc expression and memory-impairing intra-BLA infusions of lidocaine decrease Arc expression in the rACC. Western immunoblotting was used to quantify protein expression in the rACC following inhibitory avoidance training. **(A)** Significantly greater Arc protein expression was measured in the rACC of rats treated with intra-BLA infusions of clenbuterol immediately following training ($m = 1.33 \pm 0.07$, $n = 10$) when compared to vehicle-infused animals ($m = 1.00 \pm 0.05$, $n = 8$). Values are expressed as percent to normalized cage control ratios. $^*p < 0.01$. **(B)** Significantly less Arc protein expression was measured in the rACC of rats treated with intra-BLA infusions of lidocaine immediately after training ($m = 0.69 \pm 0.03$, $n = 5$) compared to vehicle-treated animals ($m = 1.00 \pm 0.10$, $n = 5$). Values are expressed as percent to normalized cage control ratios. $^*p < 0.01$.

c-FOS PROTEIN LEVELS ARE NOT INFLUENCED BY INTRA-BLA CLENBUTEROL OR LIDOCAINE INFUSIONS

In order to determine whether BLA manipulations have an effect on c-Fos protein expression, rats were trained on the IA task and were given immediate post-training intra-BLA infusions of either clenbuterol or lidocaine into one hemisphere and vehicle into the other or bilateral intra-BLA vehicle infusions. Intra-BLA infusions of clenbuterol did not significantly influence c-Fos expression (**Figure 5A**; $t(9) = 0.25$, $p = 0.40$). Similarly, intra-BLA infusions of lidocaine did not influence c-Fos protein expression in the rACC (**Figure 5B**; $t(11) = -0.67$, $p = 0.25$). These results led us to question the involvement of c-Fos in the rACC in memory for an IA task. Therefore, c-Fos protein levels were compared in tissue from the rACC of rats trained on an IA task ($n = 4$) to animals that were not trained ($n = 4$). Expression of c-Fos protein was significantly greater in the rACC of rats that were trained on the task as compared to the naïve cage control animals (**Figure 5C**; $t(4) = -2.39$, $p < 0.05$), indicating that c-Fos in the rACC is responsive to IA training, but not to manipulations of the BLA.

DISCUSSION

The main finding of these experiments is that post-training infusions of a memory-enhancing dose of clenbuterol into the BLA increase expression of the plasticity-associated proteins Arc and CaMKIIα in the rACC. Conversely, infusions of lidocaine into the BLA decrease expression of these synaptic proteins in the rACC. This is consistent with previous findings indicating that the BLA modulates Arc protein expression in the dorsal hippocampus. Taken together, these results suggest that amygdala actions may influence cellular processes involved in the storage of memory by a mechanism that is conserved, at least in part, across brain regions. Results also suggest that the effect of BLA actions is not specific to the immediate early gene Arc.

Consistent with previous findings, bilateral post-training infusions of clenbuterol into the BLA-enhanced, and bilateral intra-BLA infusions of lidocaine impaired long-term memory for the IA task (Ferry and McGaugh, 1999; Vazdarjanova and McGaugh, 1999; McIntyre et al., 2005). Using a retrograde tracer, Sripanidkulchai and colleagues demonstrated that BLA projections to the ACC are predominantly ipsilateral (1984). To examine protein expression in the current studies, the memory-modulating doses of clenbuterol or lidocaine were infused unilaterally so proteins in the rACC ipsilateral to the drug infusion could be compared to those in the rACC ipsilateral to an intra-BLA vehicle infusion. Therefore, each animal served as its own control. This paradigm is useful as individual differences in protein levels may obscure subtle group differences (McIntyre et al., 2005).

The research carried out here was based on the hypothesis that the BLA modulates the expression of proteins important for the strengthening of synapses that enables the rapid consolidation of

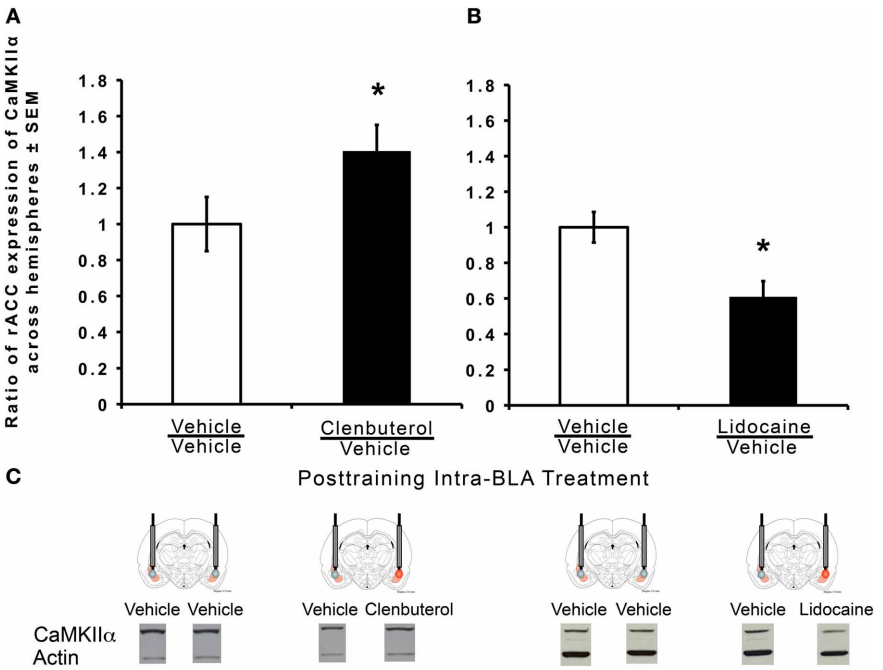

FIGURE 4 | Memory-enhancing intra-BLA infusions of clenbuterol increase CamKIIα expression and memory-impairing intra-BLA infusions of lidocaine decrease CaMKIIα expression in the rACC. Western immunoblotting was used to quantify protein expression in the rACC following inhibitory avoidance training. Values are expressed as percent to normalized cage control ratios. **(A)** Significantly greater CaMKIIα protein expression was measured in the rACC of rats treated with infusions of clenbuterol immediately following training ($m = 1.40 \pm 0.14$, $n = 5$) when compared to vehicle-infused animals (1.00 ± 0.15, $n = 6$). $^*p < 0.05$. **(B)** Significantly less CaMKIIα protein expression was measured in the rACC of rats treated with intra-BLA infusions of lidocaine immediately after training ($m = 0.61 \pm 0.09$, $n = 7$) compared to vehicle-infused animals ($m = 1.00 \pm 0.09$, $n = 7$). $^*p < 0.05$.

long-term memory of emotionally arousing events. This hypothesis is rooted in research suggesting that emotional memories can be stored for the long-term after a single experience or trial (Christianson, 1992), and the BLA interacts with multiple memory systems in the consolidation of long-term memory (McGaugh et al., 2002). Stress hormones, such as adrenaline and glucocorticoids, enhance memory consolidation in rats and humans when administered immediately after training (Gold and van Buskirk, 1975; Liang et al., 1986; Roozendaal et al., 1996; Okuda et al., 2004; Roozendaal et al., 2004; Miranda et al., 2008). Importantly, the effect of these stress hormones depends upon β-adrenoceptor activity in the BLA (Packard et al., 1995; McGaugh, 2004).

The BLA may influence rACC Arc and CamKIIα levels through effects on transport, translation, or degradation of the proteins. CamKIIα mRNA, like Arc, is found in stimulated spines of hippocampal neurons (Havik et al., 2003; Moga et al., 2004). In fact, the mRNA for both proteins is packaged in the same granule for transport to the dendrites (Gao et al., 2008). Both have internal ribosomal entry sites (IRES) that might add a translational advantage (Pinkstaff et al., 2001; Svitkin et al., 2005; Dyer et al., 2003). Finally, both Arc and CamKIIα have a 3′-UTR intron downstream of their natural stop codon, which has been shown to induce nonsense-mediated decay of Arc, potentially allowing for specific control of breakdown of the mRNAs (Giorgi et al., 2007). In contrast, c-Fos is a somatically localized protein. Consistent with previous findings that c-Fos is increased by novelty or electrical stimulation (Sagar et al., 1988), it was demonstrated here that c-Fos expression was increased in the rACC following IA training. However, post-training manipulations of the BLA did not further affect c-Fos protein expression as it did Arc and CaMKIIα. These results suggest that the increases and decreases found in Arc and CaMKIIα protein expression in rACC are not merely a result of a general increase in cellular activity due to training. The selective change in levels of locally translated proteins supports the hypothesis that the BLA modulates local translation of synaptic plasticity-associated proteins but, because protein levels were measured in lysates made from the rACC, this result does not exclude the possibility that translation occurs elsewhere in the cell. Additionally, the observed differences may mark preferential degradation of Arc and CamKIIα.

While these findings suggest that the BLA influences synaptic plasticity in the hippocampus and rACC, they do not exclude the possibility that the interactions that cause the changes in protein expression are bidirectional. Ploski and colleagues (2008) observed increases in Arc mRNA and protein expression in the lateral amygdala (LA) after auditory fear conditioning and by long-term potentiation-inducing stimulation. They further showed that the expression of Arc protein in the LA is important for long-term memory by blocking the expression of Arc protein with antisense ODN infusions into the LA (Ploski et al., 2008). Therefore, while the BLA modulates synaptic plasticity in efferent regions of the brain, transient plasticity may simultaneously

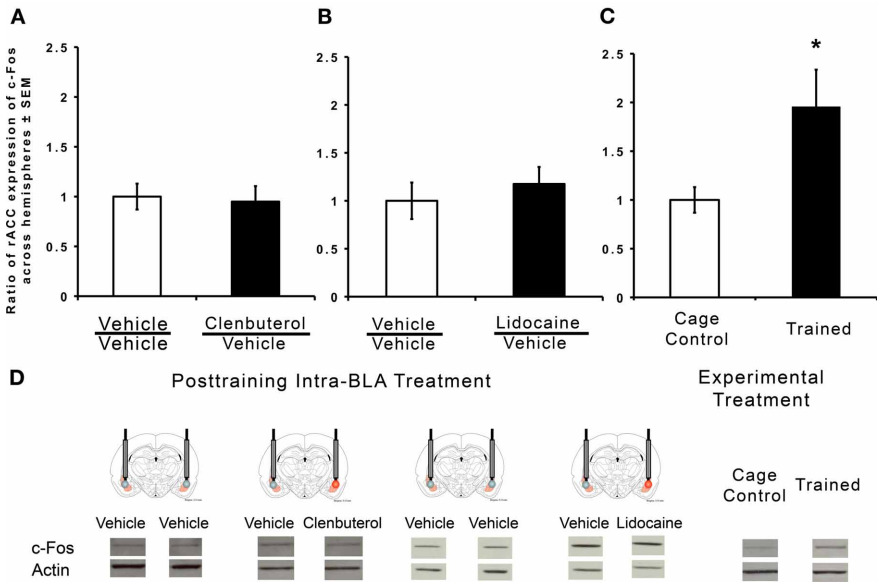

FIGURE 5 | Memory-modulating intra-BLA infusions of clenbuterol and lidocaine did not affect rACC expression of c-Fos. Western immunoblotting was used to quantify protein expression in the rACC following inhibitory avoidance training. c-Fos was normalized to actin by calculating the ratio of band density of c-Fos to that of actin, and then expressed as a percentage of normalized cage control values. **(A)** Intra-BLA infusions of clenbuterol immediately following training did not produce a significant increase in c-Fos expression in the rACC ($m = 0.94 \pm 0.15$, $n = 5$) when compared to vehicle-treated rats ($m = 1.00 \pm 0.13$, $n = 7$; $p = 0.40$). **(B)** Intra-BLA infusions of lidocaine immediately following training did not produce a significant increase in c-Fos expression in the rACC ($m = 1.17 \pm 0.17$, $n = 7$) when compared to vehicle-treated animals ($m = 1.00 \pm 0.19$, $n = 6$; $p = 0.26$). **(C)** c-Fos protein expression was significantly increased in the rACC of rats trained on the inhibitory avoidance task ($m = 1.96 \pm 0.38$, $n = 4$) when compared to untrained rats ($m = 1.00 \pm 0.13$, $n = 4$). $^*p < 0.05$.

occur within the BLA. Other regions of the brain could, likewise, influence that plasticity. For example, lesions of the BLA block the memory enhancement produced by infusions of a glucocorticoid receptor agonist into the mPFC. Phosphorylation of the extracellular-regulated protein kinase (Erk1/2) is increased within the BLA of rats treated with intra-mPFC infusions of the GR agonist and interference of this with intra-BLA infusions of a MEK inhibitor blocked the enhancement of memory produced by intra-mPFC infusions of the GR agonist (Roozendaal et al., 2009). These results suggest that the BLA and mPFC interact in a bidirectional manner to influence memory consolidation.

The present findings support the hypothesis that the BLA modulates protein expression in other areas of the brain to influence long-term memory. Stimulation of β-adrenoceptors in the BLA increases, and temporary inactivation of the BLA decreases expression of both Arc and CaMKIIα. Both proteins are critically involved in memory consolidation and synaptic plasticity. These results indicate that the modulation of Arc protein by the BLA can occur through a process that is conserved across at least two brain regions: the hippocampus and the rACC. The present results also indicate that CaMKIIα, another synaptically localized plasticity-related protein, can be modulated by pharmacological stimulation of the BLA. The finding that c-Fos was not significantly affected by the same pharmacological stimulation of the BLA suggests that activation of β-adrenoceptors in the BLA does not simply lead to a general increase in neuronal activity in the rACC. The specific changes in synaptic proteins may reflect a synapse-specific influence of the BLA on neurons in the rACC. These findings can provide a framework for understanding the mechanisms by which arousal-induced activation of the amygdala modulates consolidation of long-term memory and synaptic plasticity that is distributed across brain regions.

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Activation of nucleus accumbens NMDA receptors differentially affects appetitive or aversive taste learning and memory

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Taste memory depends on motivational and post-ingestional consequences; thus, it can be aversive (e.g., conditioned taste aversion, CTA) if a novel, palatable taste is paired with visceral malaise, or it can be appetitive if no intoxication appears after novel taste consumption, and a taste preference is developed. The nucleus accumbens (NAc) plays a role in hedonic reactivity to taste stimuli, and recent findings suggest that reward and aversion are differentially encoded by the activity of NAc neurons. The present study examined whether the requirement for *N*-methyl-D-aspartate (NMDA) receptors in the NAc core during rewarding appetitive taste learning differs from that during aversive taste conditioning, as well as during retrieval of appetitive vs. aversive taste memory, using the taste preference or CTA model, respectively. Bilateral infusions of NMDA (1 μg/μl, 0.5 μl) into the NAc core were performed before acquisition or before retrieval of taste preference or CTA. Activation of NMDA receptors before taste preference training or CTA acquisition did not alter memory formation. Furthermore, NMDA injections before aversive taste retrieval had no effect on taste memory; however, 24 h later, CTA extinction was significantly delayed. Also, NMDA injections, made before familiar appetitive memory retrieval, interrupted the development of taste preference and produced a preference delay 24 h later. These results suggest that memory formation for a novel taste produces neurochemical changes in the NAc core that have differential requirements for NMDA receptors during retrieval of appetitive or aversive memory.

Keywords: glutamate receptor, nucleus accumbens core, taste recognition, taste preference, emotional learning

INTRODUCTION

The nucleus accumbens (NAc) is a brain region located within the ventral striatum, and its critical role in processing reward-related stimuli and instrumental responses is well documented (Salamone, 1996; Corbit et al., 2001; Hall et al., 2001; Holland and Gallagher, 2003; Kelley, 2004a; Pothuizen et al., 2005). On the basis of distinctive anatomical profiles and functional specialization, the NAc has been divided into two subregions: accumbens shell and core, and their associated circuitry has also been proposed (Heimer et al., 1991; Deutch and Cameron, 1992; Zahm and Brog, 1992). Previous research suggests that the accumbens shell functions as a critical link between cortical circuits and hypothalamic/brainstem circuits with regard to the control of food intake, while the accumbens core, and its connected circuitry, is involved in the learning and execution of adaptive instrumental actions (Kelley, 2004b). The NAc participates in the control of behaviors related to natural reinforcers and is well positioned to participate in neural control of food intake, which is also related to processes required during taste memory recognition. Accordingly, the NAc has a major role in the regulation of both reward and aversion during emotional/hedonic reactivity to taste stimuli (Ventura et al., 2007; Roitman et al., 2010).

During taste learning, a taste stimulus is recognized and associated with post-ingestional consequences; an aversive taste memory is formed if intoxication or malaise signals appear, inducing a significant decrease of consumption during the next encounter with that taste. Conversely, if after consumption no intoxication is detected, an appetitive taste memory is formed, and a significant increase of consumption is induced if the taste is palatable (Núñez-Jaramillo et al., 2010). Therefore, taste learning can alter the hedonic valence of a given stimulus, and evidence suggests that the NAc has an important role during this hedonic shift (Roitman et al., 2010). Accordingly, significant changes in the release of several neurotransmitters, in both NAc subregions, have been described during learning of conditioned taste aversion (CTA; Mark et al., 1991; Hajnal and Norgren, 2001), and an increase in the expression of *c-fos* during CTA retrieval (Yasoshima et al., 2006) has also been reported. Consistent with these reports, there is evidence indicating that dopaminergic receptors in the NAc play a crucial role in regulating cell activity during reward and aversion codification of different stimuli (Carlezon Jr. and Thomas, 2009). Moreover, consumption of sucrose induces a decrease in neuronal activity, but aversively conditioned sucrose consumption induces an increase in the activity of NAc neurons (Roitman et al., 2010).

In particular, pharmacological studies have demonstrated that the NAc is needed during CTA taste memory acquisition and/or consolidation (Ramirez-Lugo et al., 2006; Yamamoto, 2007; Pedroza-Llinas et al., 2009) and that the *N*-methyl-D-aspartate (NMDA) receptors in the NAc have a modulatory role during appetitive extinction (Holahan et al., 2012). Currently, the evidence indicates that NMDA receptors in the NAc have diverse functions during some stages of taste memory formation; it has been reported that blockade of NMDA receptors in both NAc subregions impairs CTA acquisition, but this effect is stronger with NAc core NMDA blockade. Moreover, infusion of NMDA into either of the NAc subregions had no effect on attenuation of neophobia (Ramirez-Lugo et al., 2006). Taken together, the evidence suggests that NMDA receptors in the NAc have an important function during reward and/or aversive taste stimulus processing, possibly by modulating NAc dopaminergic activity (Howland et al., 2002) and interacting with other brain structures (Bermudez-Rattoni, 2004; Núñez-Jaramillo et al., 2010).

Despite such evidence, there is little information that directly compares the function of NMDA receptors in the NAc core during appetitive taste learning vs. during aversive taste conditioning, i.e., comparing their role in the different taste emotional/hedonic reactivity induced during and after learning. Thus, the present study, using the same taste stimulus in a preference protocol or CTA model, examined the requirement for NMDA receptors in the NAc core during appetitive taste learning or during CTA acquisition, and the role of these receptors during retrieval of appetitive or aversive taste memory.

EXPERIMENTAL PROCEDURES

ANIMALS

Ninety-one male Wistar rats (Instituto de Neurobiología breeding colony, Mexico; 250–300 g at time of surgery) were used. Rats were housed individually and maintained at 23°C under an inverted, 12-h/12-h light–dark cycle (lights off from 9:00 a.m. to 9:00 p.m.). All behavioral protocols were implemented during the dark portion of the cycle. Food and water were available *ad libitum* until the behavioral procedures began. Experiments were performed in accordance with the Rules in Health Matters (Ministry of Health, Mexico) and with the approval of the local Animal Care Committee.

SURGERY

All animals were anesthetized with ketamine (70 mg/kg) and xylazine (6 mg/kg) i.p. and implanted bilaterally with 12-mm, 23-gauge, stainless-steel cannulae aimed 2 mm above the NAc core (antero-posterior +1.5 mm, lateral ±1.9 mm, ventral – 4.7 mm from Bregma) according to Paxinos and Watson (1998). Cannulae were fixed to the skull with dental acrylic cement and anchored with two surgical screws placed in the skull. Stylets were inserted into the cannulae to prevent clogging. One week after surgery, animals were deprived of water for 12 h. The animals were then acclimated to the deprivation regimen for 7 days, with access to water in their home cage for 20 min each day between 12:00 and 14:00 h.

BEHAVIORAL PROCEDURES

Conditioned taste aversion

One week after surgery, the animals for the CTA experiment were water deprived and began a regime in which they had access to water from a graduated bottle for 20 min each day to establish basal water consumption. After 5 days of baseline water consumption, the CTA acquisition was implemented; animals had access to a novel 0.1% saccharin solution for 20 min, and 30 min later they were injected i.p. with lithium chloride (0.25 M LiCl, 10 ml/kg), a malaise-inducing drug (**Figure 1A**, S1). The next day, during the memory retrieval test, all rats were again exposed only to 0.1% saccharin for 20 min in order to evaluate the taste aversion (**Figure 1A**, S2). The reduction of saccharin consumption during the retrieval compared with saccharin consumption during the acquisition was used as a measure of CTA strength. Over the next 2 days, rats were again exposed to saccharin to establish extinction of CTA (**Figure 1A**, S3,S4). For data analysis, saccharin consumption during retrieval and extinction is presented as percentage of saccharin consumption during acquisition (ACQ), using the formula: % of ACQ = (milliliter of saccharin consumed during retrieval or extinction × 100)/milliliter of saccharin consumed during acquisition.

Taste preference

One week after surgery, animals were deprived of water and began a regime in which they had access to water from two graduated bottles for 20 min each day to establish basal water consumption. Bottles were placed on opposite sides of one of the narrow sides of the home cage and were rotated every 2.5 min. After 5 days of baseline water consumption, animals began the taste preference procedure. On the acquisition day, rats had access to one bottle of water and one bottle of novel 0.1% saccharin; the bottles were rotated every 2.5 min to avoid an attachment by the rat to a single bottle (**Figure 1B**, S1). The following day and 72 h later, the rats were again exposed to saccharin, with the same protocol, to evaluate the development of taste preference (i.e., appetitive taste memory retrieval; **Figure 1B**, S2,S3). Saccharin consumption is presented as a percent of the total amount of fluid consumed that day, calculated by the formula: %Sac = (milliliter of saccharin × 100)/(milliliter of saccharin + milliliter of water).

DRUGS AND INFUSION PROCEDURES

Two days before infusion, all animals were handled individually for approximately 5 min every 24 h. On infusion day, a total volume of 0.5 μl of NMDA (SIGMA, Mexico; 1 μg/μl dissolved in sterile 0.9% saline) or 0.9% saline was infused bilaterally into the NAc core, through 30-gauge injection needles connected to 10-μl Hamilton microsyringes by polyethylene tubing. Using an automated syringe pump (Sage Instruments), 0.5 μl of solution was infused bilaterally over 60 s, and the injection needle was kept in place for an additional 60 s to allow diffusion of the solution into the tissue and to minimize dragging back along the injection track. Independent groups were infused in the NAc 20 min before novel taste presentation (acquisition, **Figures 1A,B**, S1), or 20 min before the second saccharin presentation of CTA or taste preference (memory retrieval test, **Figures 1A,B**, S2).

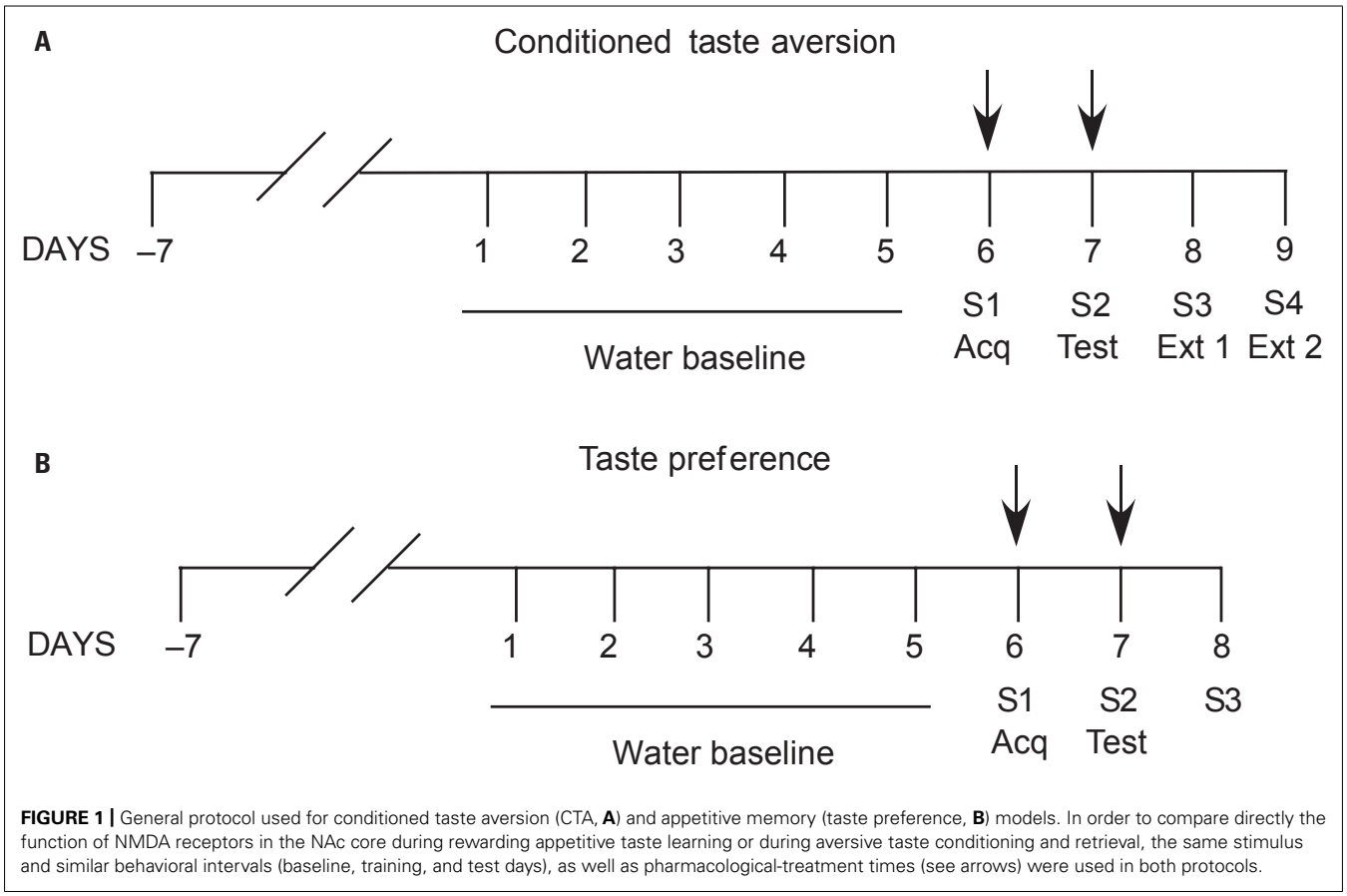

FIGURE 1 | General protocol used for conditioned taste aversion (CTA, **A**) and appetitive memory (taste preference, **B**) models. In order to compare directly the function of NMDA receptors in the NAc core during rewarding appetitive taste learning or during aversive taste conditioning and retrieval, the same stimulus and similar behavioral intervals (baseline, training, and test days), as well as pharmacological-treatment times (see arrows) were used in both protocols.

HISTOLOGY

At the end of behavioral experiments, animals were overdosed with sodium pentobarbital and perfused transcardially with 0.9% saline. The brains were removed and stored at 4°C in a 4% paraformaldehyde solution for 24 h. Brains were then floated in 30% glucose solution and stored at 4°C until they sank. Coronal sections (50 μm thick) were cut through the area of interest. The sections were stained with cresyl violet and examined microscopically to determine cannula placement (**Figure 2**). Data from animals with misplaced cannulae ($n = 14$) were excluded from the statistical analysis.

STATISTICAL ANALYSIS

In order to determine in CTA experiments, whether animals presented a change in saccharin consumption, between groups and among the 4 days of taste exposure, a repeated-measure ANOVA was performed; using the percentage of acquisition consumption [% of ACQ = (milliliter of saccharin consumed during retrieval or extinction × 100)/milliliter of saccharin consumed during acquisition], followed by *post hoc* Bonferroni/Dunn tests, where appropriate. Similarly, in the taste preference groups, a repeated-measure ANOVA was performed with the total amount of fluid consumed each day [%Sac = (milliliter of saccharin × 100)/(milliliter of saccharin + milliliter of water)]; followed by *post hoc* Bonferroni/Dunn tests, where appropriate.

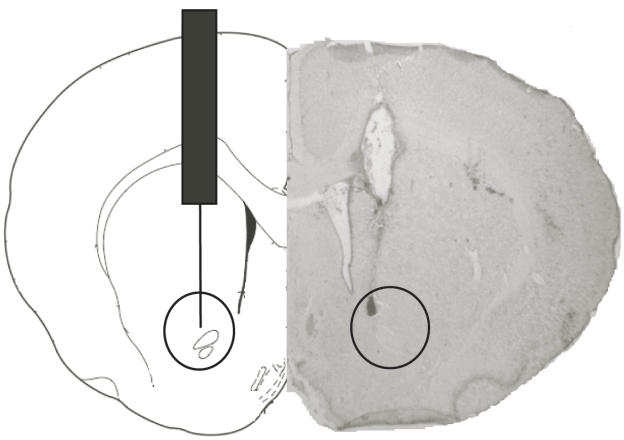

FIGURE 2 | Microphotography and diagram of coronal section of rat brain showing the area of the injection site in the NAc core.

RESULTS

NMDA RECEPTOR ACTIVATION IN THE NAc CORE BEFORE CTA ACQUISITION DOES NOT AFFECT CTA MEMORY FORMATION OR EXTINCTION

In order to evaluate the effects of NMDA receptor activation in the NAc core during CTA acquisition and/or consolidation, infusions

of NMDA were made before the first presentation of saccharin (**Figure 3A**). Repeated-measures ANOVA of percentage saccharin acquisition consumption, showed no significant differences between treatments (Saline and NMDA) groups ($F_{1,22} = 1.25$, $p > 0.05$) but revealed significant differences between treatment days (ACQ, TEST, EXT 1, and EXT 2; $F_{1,22} = 19.03$, $p < 0.01$); not significant interaction ($F_{1,22} = 1.09$, $p > 0.05$). *Post hoc* analysis showed significant differences between ACQ and TEST ($p < 0.01$), as well as between TEST and EXT 2 ($p < 0.05$) for both Saline and NMDA groups. All animals consumed similar amounts of water during baseline (data not shown). During acquisition, both Saline- ($n = 10$) and NMDA- ($n = 13$) infused groups consumed a similar amount of saccharin (11.2 ± 1.35 ml Saline and 11.69 ± 0.73 ml NMDA). As shown in **Figure 3A**, animals consumed similar percentages of saccharin during retrieval (39.4 ± 7.09% Saline and 43.45 ± 6.87% NMDA), as well as during the first (64.45 ± 7.72% Saline and 66.19 ± 8.14% NMDA) and second (108.15 ± 23.43% Saline and 81.13 ± 8.76% NMDA) extinction trials.

NMDA RECEPTOR ACTIVATION IN THE NAc CORE BEFORE RETRIEVAL PRODUCES A DELAY IN CTA MEMORY EXTINCTION

In order to evaluate the effects of NMDA receptor activation in the NAc core during aversive taste retrieval and during the extinction process, in an independent group, NMDA infusions were made before CTA test (**Figure 3B**). Repeated-measures ANOVA of percentage saccharin acquisition consumption showed significant differences between groups ($F_{1,22} = 4.51$, $p = 0.04$) and significant differences between treatment days (ACQ, TEST, EXT 1, and EXT 2) ($F_{1,22} = 40.90$, $p < 0.01$); but not significant interaction ($F_{1,22} = 1.76$, $p > 0.05$). *Post hoc* analysis showed significant differences between ACQ and TEST ($p < 0.01$), as well as between TEST and EXT 2 ($p < 0.01$) for both Saline and NMDA groups. As expected, all animals consumed similar amounts of saccharin during acquisition (Saline group, 12 ± 1.04 ml, $n = 12$; NMDA group, 13.08 ± 1.12 ml, $n = 12$), and drank similar amounts of saccharin during retrieval (Saline, 27.95 ± 5.07%; NMDA, 21.97 ± 4.85%). However, during the first extinction trial (EXT 1), *post hoc* analyses showed significant differences between Saline and NMDA groups ($p < 0.05$). The NMDA-infused group showed significantly lower saccharin consumption (44.32 ± 7.83%) than the Saline-infused group (75.62 ± 9.63%). During the second extinction trial, both groups again consumed similar amounts of saccharin (Saline, 94.89 ± 12.4%; NMDA, 78.4 ± 11.17%).

NMDA RECEPTOR ACTIVATION IN THE NAc CORE BEFORE FIRST SACCHARIN CONSUMPTION DOES NOT ALTER THE DEVELOPMENT OF TASTE PREFERENCE

In order to evaluate the effect of NMDA receptor activation in the NAc core on appetitive taste memory formation, infusions were made before the first saccharin consumption during the taste preference protocol (**Figure 4A**). All animals had similar baseline water consumption (data not shown). Repeated-measures ANOVA of percentage saccharin consumption showed no significant differences between groups ($F_{1,22} = 0.081$, $p > 0.05$) but revealed significant differences between treatment days (S1–S3; $F_{1,22} = 6.574$, $p < 0.01$); there was also a tendency to interaction

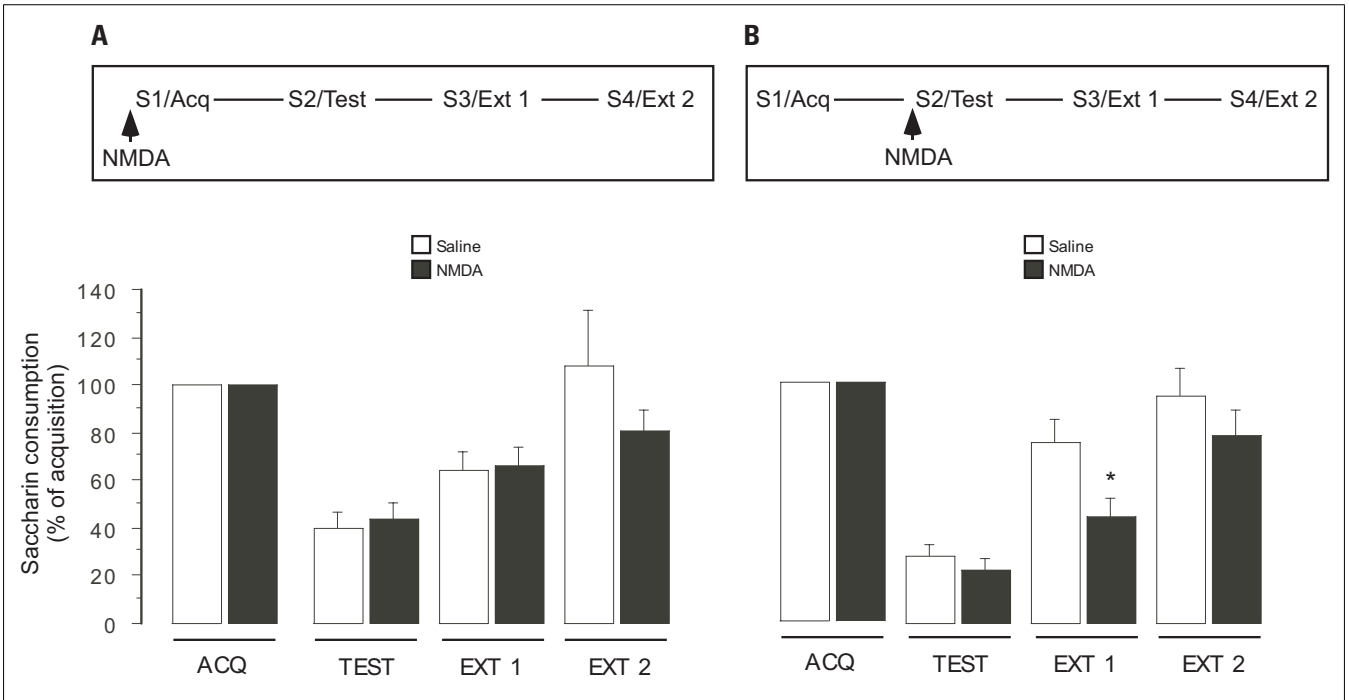

FIGURE 3 | Effects of NAc core NMDA infusion on saccharin consumption, expressed as percent of saccharin consumption during acquisition ± standard error, $*p < 0.05$, before the first (acquisition, **A**) or second (memory retrieval test, **B**) saccharin presentation during CTA. Arrows indicate the time of NAc infusion before acquisition or retrieval.

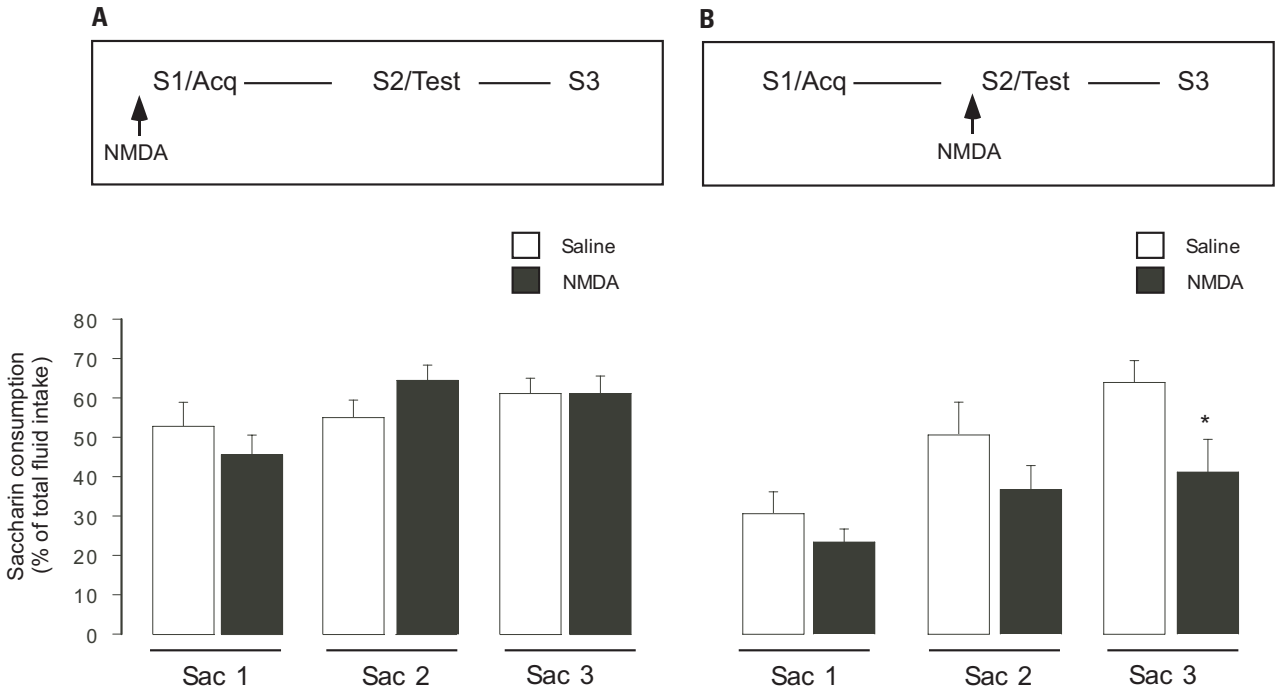

FIGURE 4 | Effects of NAc core NMDA infusion on saccharin consumption before the first (acquisition, **A**) or second (memory retrieval test, **B**) saccharin presentation during taste preference. Bars represents saccharin consumption on each day, as percent of total fluid intake ± standard error, $*p < 0.05$. Arrows at the top indicate the time of NAc infusion before acquisition or retrieval.

($F_{1,22} = 2.78$, $p = 0.07$). *Post hoc* analysis revealed that saccharin consumption changed significantly over the 3 days of saccharin exposure in the NMDA-infused group (S1–S3; $p < 0.05$), but not in the Saline-infused group ($p > 0.05$). Nevertheless, both the Saline- ($n = 11$) and NMDA- ($n = 11$) infused animals consumed comparable amounts of saccharin during the first (52.65 ± 6% Saline group and 45.3 ± 5.25% NMDA group), second (54.86 ± 4.46% Saline and 64.2 ± 3.86% NMDA), and third (61.24 ± 3.7% Saline and 60.96 ± 4.48% NMDA) saccharin presentation (**Figure 4A**).

NMDA RECEPTOR ACTIVATION IN THE NAc CORE BEFORE SECOND SACCHARIN CONSUMPTION DISRUPTS TASTE PREFERENCE

To evaluate the effect of NMDA receptor activation in the NAc core during retrieval of appetitive taste memory, infusions were done before the second saccharin presentation during the taste preference protocol (**Figure 4B**). All animals consumed similar amounts during baseline water consumption (data not shown). During the first saccharin consumption, animals of the Saline- ($n = 11$) and NMDA- ($n = 11$) infusion groups consumed similar amounts of saccharin (30.52 ± 5.5% Saline and 23.3 ± 3.18% NMDA; **Figure 4B**). Repeated-measures ANOVA of the percentage saccharin consumption showed significant differences between groups ($F_{1,22} = 5.156$, $p < 0.05$) and also revealed significant differences between treatment days (S1–S3; $F_{1,22} = 11.54$, $p < 0.01$). Saccharin consumption increased significantly over the 3 days of saccharin exposure (S1–S3) in the Saline-infused animals ($p < 0.01$), but not in the NMDA-infused animals ($p > 0.05$). *Post hoc* test revealed no significant difference between groups during first saccharin consumption, either significant difference between groups during second day consumption ($p = 0.07$; 50.62 ± 8.08% Saline and 36.55% NMDA). During the third saccharin exposure the NMDA-infused group had significantly ($p < 0.05$) lower saccharin consumption (41.13 ± 8.36%) than the Saline-infused group (63.95 ± 5.62%; **Figure 4B**).

DISCUSSION

The present results demonstrated that NMDA receptor activation in the NAc core has differential effects on taste memory formation and retrieval, depending on whether an appetitive or aversive memory is formed. NMDA receptor activation before acquisition of aversive taste memory had no effect on memory formation. Moreover, NMDA receptor activation before aversive memory left retrieval intact, but significantly delayed the extinction of taste aversion (**Figure 3**). The results also showed that the NAc core is a sensitive structure during taste preference acquisition, since saline infusion during acquisition did influence taste consumption, compared with the experimental group that received saline infusion during retrieval, or compared with an intact animals (data not shown), that consumed approximately 30% less saccharin during the first day of the taste preference protocol. Despite this effect, the total volume of saccharin consumed during the first presentation did not differ between groups infused either with saline or NMDA. Moreover, the saline infusion before

the first saccharin consumption did not impair the formation of an appetitive taste memory, since animals consumed a large amount of saccharin during the second exposure to saccharin, compared with the intact group or with the group infused with saline during retrieval (see **Figure 4B**). These data suggest that saline infusion into the NAc core before the first saccharin consumption did not affect either taste perception or the formation of taste appetitive memory; possibly, it altered drink motivation or the ability to detect saliency between stimuli (e.g., water vs. saccharin). In this regard, previous evidence showed that blockade of NMDA receptors induces deficits in signaling processes that participate in termination of satiety signals (Burns and Ritter, 1997), and inhibition of some NAc regions changes the positive hedonic responses to palatable taste solutions (Pecina and Berridge, 2005). Furthermore, the data obtained during CTA acquisition corroborates this idea, because saline or NMDA infusions, made before the first saccharine consumption, had no effect on taste memory formation, which requires an intact taste perception. Further studies are needed to understand the NAc core involvement during processing of relevant properties in the first taste encounter.

Our results also show that NMDA receptor function differs between acquisition and retrieval of taste appetitive memory, since NMDA receptor activation before memory retrieval significantly delayed the development of taste preference after the second saccharin consumption (**Figure 4B**). Previously, it was reported that blockade of NMDA receptors in the NAc does not affect attenuation of neophobia, another model of appetitive taste memory (Ramirez-Lugo et al., 2006). However, there are important differences between attenuation of neophobia and the taste preference protocol; during attenuation of neophobia only one bottle was presented, while in taste preference, subjects were able to choose between saccharin and water. Another difference is that the saccharin concentration used in the attenuation of neophobia (0.5%) was greater than the concentration used in the current taste preference task (0.1%).

As mentioned earlier, since taste memory formation depends on motivational and post-ingestional consequences, the memory formed after novel taste consumption can be associated with a positive reward, or with an aversive consequence, as observed during CTA (Bermudez-Rattoni, 2004; Núñez-Jaramillo et al., 2010). Consequently, taste learning can alter the hedonic valence of a given stimulus (Gutierrez et al., 2003; Bermudez-Rattoni, 2004), which could be related to the evidence that NAc has a role in the assignment of hedonic value of stimuli, either positive or negative (Ventura et al., 2007; Roitman et al., 2010). Accordingly, it could be argued that NMDA receptor activation, before retrieval of either appetitive or aversive taste memory, increases the negative hedonic value of the previous taste memory representation. In line with this, our results demonstrate that consumption of familiar saccharin (third presentation), either appetitive or aversive, significantly decreases after NMDA activation, diminishing preference or extinction.

The present data could be also related to electrophysiological findings that consumption of palatable food stimuli is associated with decreases in the firing rate of the majority of responsive NAc neurons (Nicola et al., 2004; Roitman et al., 2005; Taha and Fields, 2005; Wheeler et al., 2008) and that the activity of NAc cells decreases during rewarding stimulus presentation and increases in the presence of aversive stimulus (Carlezon Jr. and Thomas, 2009; Roitman et al., 2010). We found that NMDA receptor activation correlates mainly with retrieval or memory processing of familiar appetitive or aversive taste. Although NMDA receptor activation did not affect CTA retrieval, it did produce a significant delay in aversive extinction (**Figure 3B**), indicating that the NAc core has an important role during the process of re-learning an aversive stimulus. Taken together, these results suggest that activation of NAc core NMDA receptors before retrieval of either appetitive or aversive taste memory enhances the aversive value associated with the taste.

The current results suggest that taste learning induces neurochemical changes in the NAc core during memory retrieval and re-learning of the same stimulus; these changes lead to a differential taste processing that is modulated by the glutamatergic system. In this regard, it has been reported that taste learning produces important biochemical changes in various brain structures such as the insular cortex, amygdala, and NAc (Bermudez-Rattoni, 2004; Núñez-Jaramillo et al., 2010). Thus, the differential effect of NMDA receptor activation on taste memory acquisition or retrieval, reported here, might be due to a reorganization of the neurochemical processes in the NAc that occurs as a consequence of taste memory formation. This reorganization could involve changes in the localization of glutamate receptors, as previously reported in other structures (Heynen et al., 2000; Goebel et al., 2005; Núñez-Jaramillo et al., 2008) and modulation of systems using other neurotransmitters, such as dopamine and acetylcholine (Mark et al., 1991, 1995; Howland et al., 2002). Further studies are needed to confirm that memory formation produces plastic changes in the NAc core that induce differential requirements for NMDA receptors during appetitive or aversive taste learning and memory. Our results also give evidence that the NAc core is a sensitive structure during taste appetitive memory formation, since control animals with infusions during the first day of taste preference training did not decrease their saccharin consumption. These data suggest a modulatory role of the NMDA receptors during drink motivation or the ability to detect saliency between stimuli (e.g., water vs. saccharin), that could be observed by the preference (two bottles) protocol used in these experiments.

In conclusion, the present study directly compares the function of NMDA receptors in the NAc core during appetitive taste learning or during aversive taste conditioning. Using the same taste stimulus in a preference protocol or CTA model, we provide evidence of an important regulatory role for NMDA receptors in the NAc core during memory retrieval and re-learning of rewarded or aversive taste.

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Encoding of emotion-paired spatial stimuli in the rodent hippocampus

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Rats can acquire the cognitive component of CS-US associations between sensory and aversive stimuli without a functional basolateral amygdala (BLA). Thus, other brain regions should support such associations. Some septal/dorsal CA1 (dCA1) neurons respond to both spatial stimuli and footshock, suggesting that dCA1 could be one such region. We report that, in both dorsal and ventral hippocampus, different neuronal ensembles express immediate-early genes (IEGs) when a place is experienced alone vs. when it is associated with foot shock. We assessed changes in the size and overlap of hippocampal neuronal ensembles activated by two behavioral events using a cellular imaging method, *Arc/Homer1a* catFISH. The control group (A-A) experienced the same place twice, while the experimental group (A-CFC) received the same training plus two foot shocks during the second event. During fear conditioning, A-CFC, compared to A-A, rats had a smaller ensemble size in dCA3, dCA1, and vCA3, but not vCA1. Additionally, A-CFC rats had a lower overlap score in dCA1 and vCA3. Locomotion did not correlate with ensemble size. Importantly, foot shocks delivered in a training paradigm that prevents establishing shock-context associations, did not induce significant *Arc* expression, rejecting the possibility that the observed changes in ensemble size and composition simply reflect experiencing a foot shock. Combined with data that Arc is necessary for lasting synaptic plasticity and long-term memory, the data suggests that *Arc/H1a*+ hippocampal neuronal ensembles encode aspects of fear conditioning beyond space and time. Rats, like humans, may use the hippocampus to create integrated episodic-like memory during fear conditioning.

Keywords: Arc, Homer 1a, fear conditioning, emotional memory, ventral CA1, ventral CA3, catFISH, dorsal hippocampus

INTRODUCTION

Forming associations between neutral and aversive stimuli is essential for survival. Such learning can be conceptualized in classical conditioning terms as forming CS-US associations, where a conditioned stimulus (CS) comes to elicit conditioned emotional responses only after being paired with an unconditioned stimulus (US), which elicits unconditioned emotional responses before the pairing (Pavlov, 1927). Although experimentally, responses are measured and used to assess the strength of associations, classical conditioning training elicits at least two types of learning: forming stimulus-response (S-R) associations and forming stimulus-stimulus (S-S) associations.

Conceptually, the existence of what is now known as "stimulus-stimulus associations" was postulated by Tolman (Tolman, 1949). It is revealed by latent learning and sensory preconditioning, and has been demonstrated for contextual fear conditioning (Tolman, 1949; Vazdarjanova and McGaugh, 1998; O'Brien and Sutherland, 2007; Li et al., 2011). Associations between emotional and sensory stimuli can be assessed by measuring behaviors that are supported by such associations, but which were not conditioned during the training. Rats can flexibly express an acquired association between a shock and a place, independent from any reinforced responses during the training. When rats with pre- or post-training lesions of the basolateral amygdala (BLA) are confined to one context during training and receive an aversive stimulus (contextual fear conditioning), but are allowed to make a choice between this context over a context where they have not received foot shocks during testing, they avoid the footshock-associated context, even when they show little freezing (Vazdarjanova and McGaugh, 1998, 1999). Therefore, structures other than the BLA must support the flexible expression of footshock-place associations.

Flexible expression of stimulus-stimulus associations is a hallmark of episodic-like memories which are known to exist in non-human animals, and to rely on associating "what," "where" and "when" of an experience (Clayton and Dickinson, 1998; Morris, 2001; Babb and Crystal, 2006; Ferbinteanu et al., 2006; Kart-Teke et al., 2006; Clayton et al., 2007; Eichenbaum et al., 2011). Such associations are supported by the hippocampus (Morris, 2001; Bast et al., 2005; Li and Chao, 2008; Sauvage et al., 2008), likely in conjunction with closely related structures, such as the entorhinal and perirhinal cortex, as well as the BLA and prefrontal

cortex. In humans, S-S associations for emotional stimuli during classical conditioning depend on the hippocampus (Bechara et al., 1995) and are modulated by the amygdala (Cahill and McGaugh, 1998). In animals, episodic-like memories have been demonstrated during fear conditioning (O'Brien and Sutherland, 2007). Fear conditioning also changes the firing characteristics and "place fields" of dCA1 cells (Moita et al., 2004; Chen et al., 2009). Whether the reported transient firing is sufficient to induce molecular changes that support long-term memory, or whether such changes are intrinsic to the dCA1 is unknown. Furthermore, it is unknown whether the ventral/temporal, compared to the dorsal/septal, part of the hippocampus preferentially contributes to such changes. Due to its connectivity, distinct gene expression patterns, and evidence from lesion and pharmacological studies, the ventral hippocampus has been associated with the temporal lobe system which regulates motivational and emotional behavior, while the dorsal hippocampus has been associated with navigation and exploration (reviewed in Fanselow and Dong, 2010; Bast, 2011). Therefore, we investigated whether changes during fear conditioning, independent of spatial cues, are encoded by the CA1 and CA3 hippocampal regions and whether they are more pronounced in the ventral, as compared to the dorsal, hippocampus.

We assessed large neuronal ensembles from the dorsal and ventral parts of the CA1 and CA3 regions of the hippocampus using a cellular imaging method which examines plasticity-related immediate-early gene (IEG) expression: *Arc/H1a* catFISH (cellular compartment analysis of temporal activity by fluorescence *in-situ* hybridization using *Arc* and *Homer 1a*) (Vazdarjanova et al., 2002). *Arc/Arg3.1* (activity-regulated cytoskeleton-associated protein) and *Homer1a (H1a)* are effector IEGs that are coordinately expressed during behavior (Vazdarjanova et al., 2002) and are useful markers of plasticity. The proteins they encode are targeted to, or synthesized at, activated dendrites, where they participate in establishing structural and functional changes that support homeostatic scaling, long-term synaptic plasticity, and long-term memory (reviewed in Fagni et al., 2002; Szumlinski et al., 2006; Bramham et al., 2010). Importantly, *Arc* expression can be dissociated from activity and predicts the pattern of plasticity assessed electrophysiologically one day later (Guzowski et al., 2006; Carpenter-Hyland et al., 2010).

The *Arc/H1a* catFISH method is a powerful tool that can detect differences in the size and overlap of large neuronal ensembles, which have initiated expression of *Arc/Arg3.1* and *Homer1a* during two temporally separate behavioral episodes, across multiple brain regions in the same animals. Due to the brief period of transcription (<10 min) of these genes and the differences in size between their primary transcripts, *H1a* intranuclear foci of transcription mark cells that initiated transcription 25–35 min before the animal's death, while those with *Arc* foci mark cells with transcription initiated 2–12 min before death (Guzowski et al., 1999; Vazdarjanova et al., 2002). Using this method it has been shown that exploring the same place twice induces *Arc/H1a* expression in largely overlapping dCA1 neuronal ensembles (Guzowski et al., 1999; Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004; Ramírez-Amaya et al., 2005), similar to findings from electrophysiological studies of "place cells" (McNaughton et al., 1989; Barnes et al., 1990; Lee et al., 2004; Leutgeb et al., 2004). Here we show that both the size and overlap of hippocampal IEG-expressing neuronal ensembles is decreased by fear conditioning, compared to experiencing the same place with the same spatial features and relationships. We also show that the changes vary between CA3 and CA1 and along the septo-temporal axis of the hippocampus.

MATERIALS AND METHODS

SUBJECTS

Young adult (250–300 g) male Sprague-Dawley rats (Charles Rivers Laboratories Inc., MA) were double housed on a 12 h light/dark cycle (lights on at 7:00 am) with food and water freely available. All testing was performed between 9:00 am and 5:00 pm.

BEHAVIORAL PROCEDURES

All behavioral procedures were approved by the Institutional Animal Care and Use Committee (IACUC), Georgia Health Sciences University, Augusta, GA. All analyses were performed by an experimenter unaware of the group assignment of each rat.

EXPERIMENTAL DESIGN

The experimental design is summarized in **Figure 1A**. A-A animals ($n = 6$) were subjected to two emotionally neutral (no shock) explorations of a $50 \times 10 \times 19$ cm box, made of two stainless steel plates separated at the floor with a 1 cm gap. The two, 5 min explorations were separated by a 20 min rest period in the home cage. The A-CFC animals ($n = 6$) received

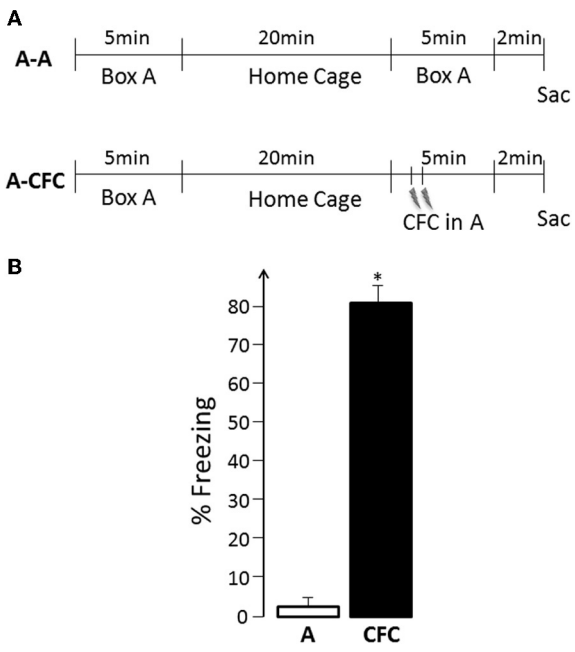

FIGURE 1 | (A) Experimental Design: A, Exploration of the experimental box (no shock); CFC, Contextual fear conditioning in the same box; Sac, Sacrifice; **(B)** Freezing behavior during the second event- spatial exploration vs. fear conditioning, $*p < 0.001$.

identical treatment, except that during the second exploration they received two foot shocks administered through the stainless steel plates (1 mA AC, 1 s, 30 s apart, first shock 1 min into the session). An additional 12 rats received either CFC training or spatial exploration and, 24 h later, were placed in the apparatus for 5 min to assess long-term memory of the training. No foot shocks were administered during the testing. All behavior was recorded using an overhead camera. Fear behavior was measured as time spent freezing, which was scored as lack of movement except that needed for respiration. A crossing was scored when a rat was moving through the apparatus and the base of the tail crossed the midpoint of the apparatus. To assess the percentage of cells with IEG expression in response solely to foot shock, three rats were introduced to the apparatus, immediately shocked for 2 s, and then promptly removed and anesthetized with isoflurane to prevent IEG expression due to subsequent exploratory behavior that is usually seen with such "immediate shock" treatment. These rats were maintained under general anesthesia for 5 min to allow footshock-initiated transcription to proceed, and then the brain was harvested. Three more rats were used as "cage controls." They were taken directly from their cage, anesthetized and the brains were harvested.

BRAIN HARVESTING AND SECTIONING

At the end of each experiment, the animals were decapitated using a rat guillotine, the brain rapidly harvested, flash-frozen in isopentane, and stored at –80°C. Right hemispheres of brains from different conditions were blocked together in freezing medium such that several behavioral groups were represented in each block. Twenty micron thick coronal sections were cut on a cryostat and mounted on glass slides. The slides were stored at –20°C until further processing.

Arc/H1a catFISH

Slides were processed for double *Arc/H1a in situ* hybridization as described previously (Vazdarjanova and Guzowski, 2004). Briefly, after fixing and permeabilizing the tissue, a fluorescein-labeled full-length *Arc* antisense riboprobe and digoxigenin-labeled *H1a* antisense riboprobe, targeting the 3′ UTR of *H1a* mRNA, were applied and hybridized overnight at 56°C. Following quenching of peroxidase activity, the fluorescein tag was revealed with peroxidase-conjugated anti-fluorescein antibody (Fab fragments, Roche, Indianapolis, IN) and a tyramide amplification reaction with either fluorescein or cyanine 3 (SuperGlo™ Green and SuperGlo™ Red, Fluorescent Solutions, Augusta, GA); these steps were repeated using peroxidase-conjugated anti-digoxigenin antibody (Fab fragments, Roche, Indianapolis, IN). Riboprobes were generated using MaxiScript (Ambion, Austin, TX) and AmpliScribe (Epicentre Biotechnologies, Madison, WI) *in vitro* transcription kits and fluorescein- and digoxigenin-labeled UTP (Roche). Nuclei were counterstained with DAPI.

IMAGE ACQUISITION AND STEREOLOGICAL ANALYSIS

Image stacks from the selected regions (2.8–3.8 mm and 4.8–6.4 mm posterior to bregma for dorsal and ventral hippocampus, respectively) were collected from at least four different slides per animal with a 20× objective on a Zeiss AxioImager/Apotome system. For the dCA3 region, mosaic images of CA3b were collected and analyzed. The position of the dCA1, vCA1, and vCA3 are illustrated in **Figures 2A** and **D**. The choice of imaging fields from the ventral hippocampus was based on the projection patterns of the BLA (Pikkarainen et al., 1999; Petrovich et al., 2001).

Unbiased stereological cell counting and classification were performed as follows: (1) neurons were first segmented from the appropriate regions of interest; (2) segmented neurons were classified manually using AxioVision imaging software (Zeiss). Putative glial cells, those with small, intensely, and uniformly stained nuclei, were excluded from the analysis. Neuron-like cells in the regions of interest were counted by using an optical dissector method which minimizes sampling errors attributable to partial cells and stereological concerns (West et al., 1991). Cells were classified as *Arc+*, *H1a+*, *or Arc/H1a+* depending on whether they had foci of transcription for *Arc, H1a*, or both, respectively. Those cells that lacked foci were classified as negative. Overlap scores were calculated as: $(\%\text{Arc/H1a} + (\%E_1 \times \%E_2)/100)/(\%E_{min} - (\%E_1 \times \%E_2)/100)$, where %Arc/H1a+ = percentage of total neurons that are *Arc/H1a+*; $\%E_1$ = percentage of cells activated in the first behavioral event = (*H1a+* + *Arc/H1a+*)/total neurons; $\%E_2$ = percentage of cells activated in the second behavioral event = (*Arc+* + *Arc/H1a+*)/total neurons; $(\%E_1 \times \%E_2)/100$ = theoretical probability of cells activated during both events, assuming the two events activated independent groups of neurons; $\%E_{min}$ = lesser of $\%E_1$ and $\%E_2$, which normalizes the equation to a condition with a perfect overlap even if one population is smaller than the other. The Overlap Score is 1 for a perfect overlap ($\%E_1 = \%E_2$), and is 0 for two statistically independent $\%E_1$ and $\%E_2$. For purposes of clarity we refer to all *H1a+* and *Arc/H1a+* cells as an "ensemble" that activated IEG expression during the first event, and all *Arc+* and *Arc/H1a+* cells as an ensemble that activated IEG expression during the second event.

STATISTICAL ANALYSES

Time spent freezing, number of crossings, and overlap scores in the control and experimental groups were compared using analysis of variance (ANOVA). Ensemble size activated by the different events within the same control and experimental animals was analyzed using repeated measures ANOVA with Event and Group as the independent variables and ensemble size as the dependent variable. Statistically significant main effects were followed up by Fisher's PLSD *post-hoc* tests for four *a-priori* set comparisons: Event 1 vs. Event 2 for the A-A and A-CFC groups and A-A vs. A-CFC for Event 1 and Event 2. Correlation of ensemble size between regions was analyzed with simple regression analyses (StatView software, SAS Institute). Differences were considered statistically significant at $p < 0.05$.

RESULTS

FOOT SHOCK DURING A SECOND VISIT TO A NEW PLACE INDUCED FEAR CONDITIONING

The experimental design is illustrated in **Figure 1A**. During the first exposure to the training apparatus, all rats displayed active exploration (mean of 11 crossings, range 6–13, for both the A-A and A-CFC groups, no difference between the two groups)

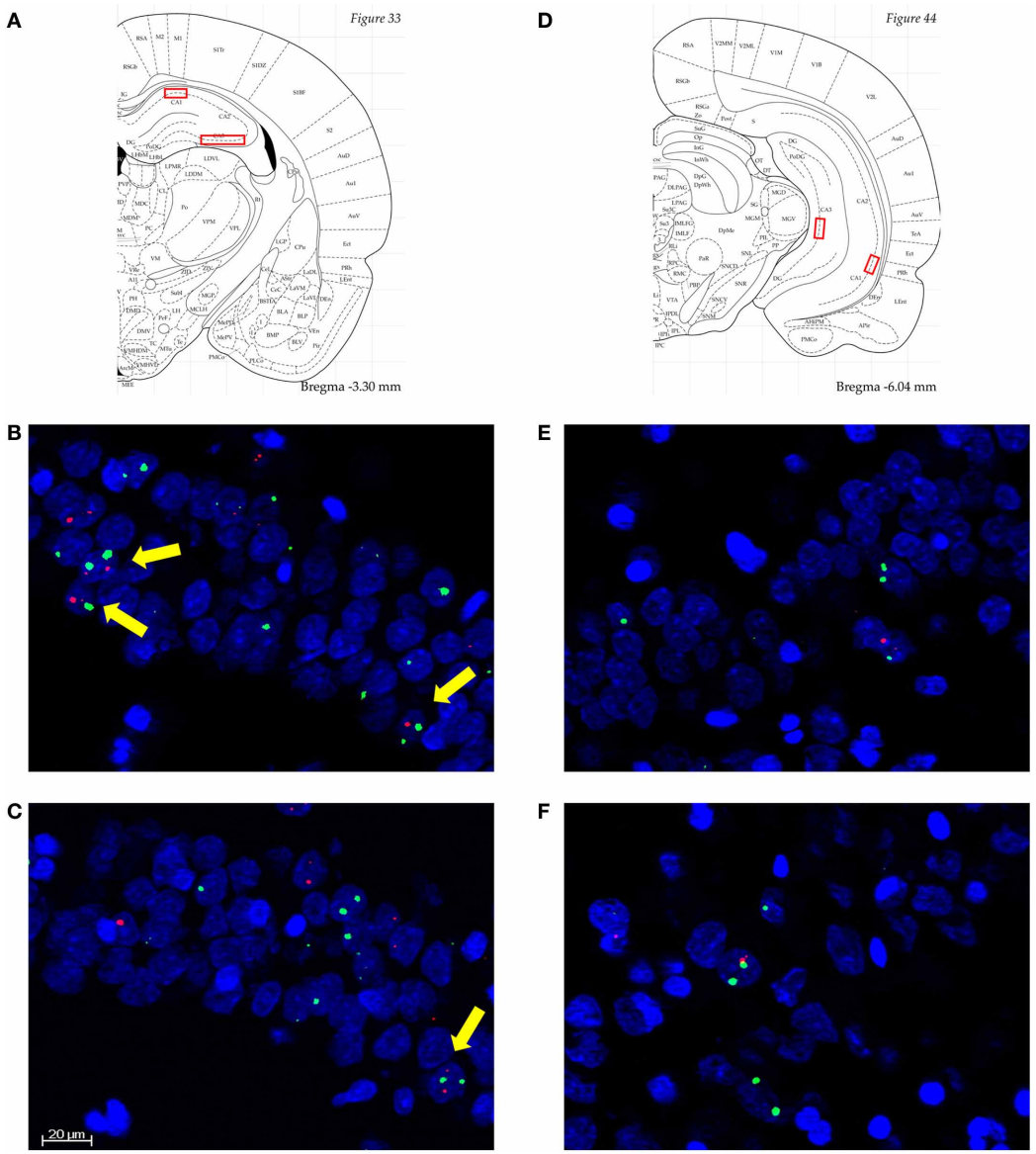

FIGURE 2 | (A) and **(D)** Diagrammatic representation of the approximate anatomical locations from which the analyzed images were collected (Paxinos and Watson, 4th Edn, Academic Press, San Diego, 1998). Septal/dorsal CA1 (dCA1), temporal/ventral CA1 (vCA1), and vCA3 were single stacks, while dCA3 were mosaics through dCA3b. **(B)**–**(F)** Representative images from dCA1 of an A-A rat **(B)**; dCA1 of an A-CFC rat **(C)**; vCA1 of an A-A rat **(E)**; and vCA1 of an A-CFC rat **(F)**. *Arc* is in red, *H1a* is in green, and nuclei are in blue. Scale bar is 20 μm. All images are 3 μm thick. Note the higher number of neurons with *Arc/H1a* foci of transcription, to which yellow arrows point, in the A-A compared to A-CFC rats. The images also illustrate the lower percentage of IEG-expressing neurons in the vCA1, compared to the dCA1.

and virtually no freezing. A-A rats showed similar behavioral patterns during their second visit to the same place. While A-CFC rats did not show freezing behavior before the delivery of the two foot shocks, there was robust post-shock freezing compared to the freezing displayed by the A-A rats during the same period of time [$F_{(1,\ 10)} = 226.38$, $p < 0.0001$, **Figure 1B**]. Thus, A-CFC rats acquired robust contextual fear conditioning, as evidenced by their continued display of high levels of fear when the aversive stimulus itself was no longer present. Such CFC training also resulted in long-lasting memory for the event: A-CFC rats tested 24 h later showed significantly more freezing than A-A rats (mean 62% vs. 2%, respectively, $F_{(1,\ 10)} = 33.18$, $p < 0.001$).

PAIRING FOOT SHOCK WITH SPATIAL STIMULI INDUCED A LARGE DECREASE IN THE SIZE OF DORSAL/SEPTAL CA1 (dCA1) NEURONAL ENSEMBLES EXPRESSING PLASTICITY-RELATED IMMEDIATE-EARLY GENES (IEGs)

Images collected from dCA1 (**Figure 2A**) revealed that both spatial exploration (**Figure 2B**) and fear conditioning in the same context (**Figure 2C**) induced significant expression of *Arc* and *H1a*. While caged control rats had a very low percentage of *H1a*+

or *Arc*+ cells (1% and 3%, respectively), Event 1 induced similarly high IEG expression in both A-A and A-CFC rats (**Figure 3A**). Visiting the same place a second time led to a decrease in the size of IEG+ ensembles (main effect of Event: $F_{(1,\ 10)} = 38.88$, $p < 0.0001$). The decrease was relatively mild in the A-A group ($p = 0.047$) and very pronounced in the A-CFC group ($p = 0.001$). The CFC training appears to account for the decreased ensemble size in the A-CFC group, as revealed by a significant Event × Group interaction [$F_{(1,\ 10)} = 5.24$, $p < 0.05$] and a significant difference between the A-A and A-CFC groups during Event 2 ($p = 0.018$). The decrease could not be explained by the fact that the A-CFC rats were moving less and showing more freezing behavior, because there was no correlation between the percentage of cells activated during the CFC training (*Arc*+ neurons) and the number of crossings during the training ($b = -0.06$, $R^2 = 0.24$, ns); nor was there a correlation between the number of crossings during the first exploration and the percentage of *H1a*+ neurons ($b = +0.09$, $R^2 = 0.007$, ns). The overall proportions of neurons expressing *Arc/H1a* during each event was smaller than previously observed with the catFISH method (Guzowski et al., 1999; Vazdarjanova et al., 2002, 2006; Vazdarjanova and Guzowski, 2004; Ramírez-Amaya et al., 2005) which is likely due to the much smaller size of the current apparatus (*x*, *y* dimensions: 50 × 10 cm), with fewer spatial cues, than in the referenced publications (i.e., 61 × 61 cm).

FEAR-CONDITIONING-INDUCED PARTIAL "REMAPPING" IN dCA1

Not only were fewer dCA1 neurons activated during CFC, compared to exploring the same place without the aversive event, but the majority of the CFC-activated neurons were different from those activated by exploring the place alone: the overlap score for dCA1 in A-CFC rats was significantly smaller than the overlap score of A-A rats (**Figure 3B**, $F_{(1,\ 10)} = 5.00$, $p < 0.05$). This partial "remapping," to borrow a term from neuroelectrophysiology, is not a function of the decreased size of the neuronal ensembles during CFC, because the overlap score is adjusted for the smaller of the two behavioral events (see "Materials and Methods"). Additionally, only 23.1% of the cells activated during Event 1 (*H1a*+ cells) were also activated during Event 2 in the A-CFC group (*Arc*+/*H1a*+ cells), compared to 44.2% for the A-A group [$F_{(1,\ 10)} = 15.31$, $p < 0.01$]. Graphically, the relationships between size and overlap of the ensembles activated in the first and second event are illustrated with Venn diagrams in **Figure 3C**. Note that although the overall percentage of IEG+ cells activated by both events is similar for the A-A and A-CFC groups, there is a big difference in the overlap. This change in overlap does not appear to be a simple reflection of experiencing a foot shock, because foot shock alone induced *Arc* in very few dCA1 neurons (4% vs. 3% in caged controls).

FEAR-CONDITIONING-INDUCED CHANGES WERE LESS PRONOUNCED IN dCA3

Fear-conditioning-induced changes in dCA3 ensembles were similar, but less pronounced, than those seen in dCA1. The ensemble size during the second event was smaller than that of the first event for both the A-A and A-CFC groups (Event effect: $F_{(1,\ 10)} = 36.78$, $p < 0.0001$; and only a trend of a group effect $p = 0.09$, **Figure 4A**). However, the neuronal ensemble activated by Event 2 was smaller in the A-CFC versus the A-A rats ($p < 0.05$). Additionally, compared to exploration alone, CFC activated fewer of the neurons activated by the first exploration (A-A= 50.1% vs. A-CFC= 31.1%, $p = 0.013$, **Figure 4C**). The overlap score was

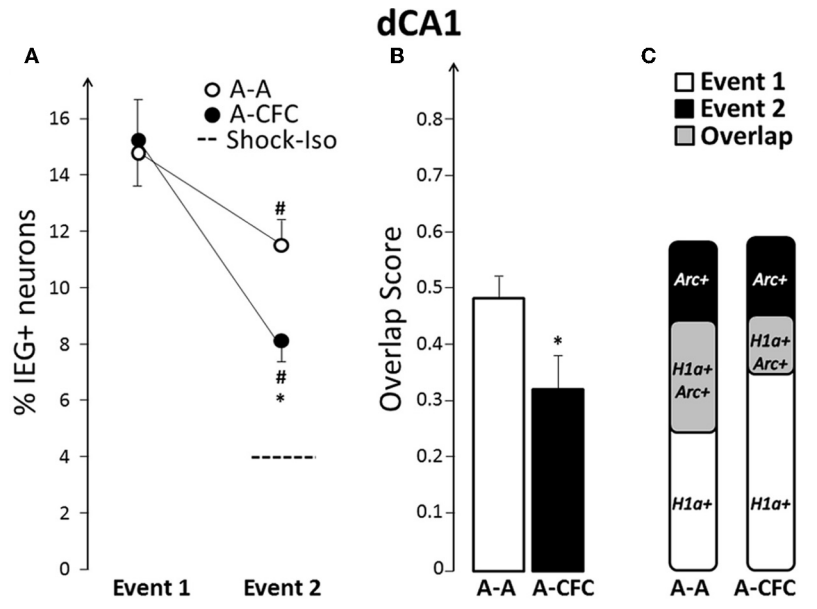

FIGURE 3 | Comparison of ensemble size and overlap between dCA1 ensembles activated during both events in A-A and A-CFC groups: (A) Size of IEG-expressing ensembles expressed as percentage of total cells. The dashed line is the percentage of dCA1 cells expressing *Arc* in response to foot shock alone. **(B)** Overlap scores; **(C)** Venn diagrams representing both size and overlap. $^{*}p < 0.05$ compared to the A-A group; $^{\#}p < 0.05$ compared to Event 1 of the same group.

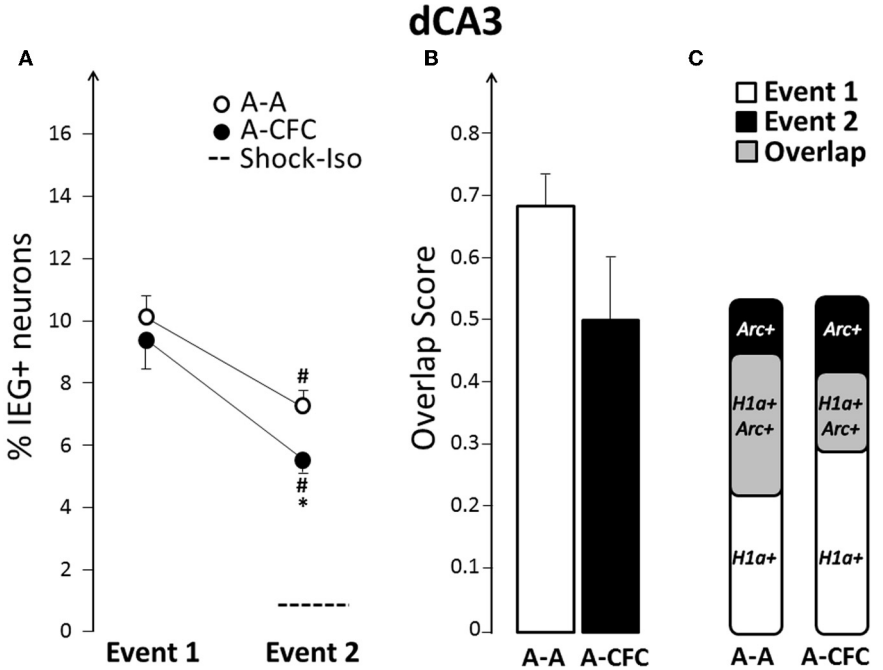

FIGURE 4 | Comparison of ensemble size and overlap between dCA3 ensembles activated during both events in A-A and A-CFC groups: (A) Size of IEG-expressing ensembles expressed as percentage of total cells. The dashed line is the percentage of dCA3 cells expressing *Arc* in response to foot shock alone. **(B)** Overlap scores; **(C)** Venn diagrams representing both size and overlap. $^{*}p < 0.05$ compared to the A-A group; $^{\#}p < 0.05$ compared to Event 1 of the same group.

also decreased for the A-CFC group (**Figure 4B**), although it did not reach statistical significance due to a high overlap score in one A-CFC rat (without this rat, $p = 0.028$). Interestingly, the same rat had the lowest overlap score in dCA1. Such negative correlation between overlap scores in the dCA1 and dCA3 was observed for the entire A-CFC group ($b = -0.46$, $R^2 = 0.65$, $p = 0.05$), while no correlation was present in the A-A group ($b = +0.48$, $R^2 = 0.40$, $p = 0.18$).

NO CFC-INDUCED CHANGES IN SIZE OR OVERLAP IN vCA1

On the basis of findings that functional integrity of, or plasticity in, the ventral hippocampus influences fear conditioning (Zhang et al., 2001; Maren and Holt, 2004; Hunsaker and Kesner, 2008); we hypothesized that there will be a more pronounced change in size and/or overlap in vCA1 and vCA3, compared to their dorsal counterparts. In contrast to this hypothesis, there was no difference in either the ensemble size (group effect: $F_{(1,\ 10)} = 2.76$, ns), or overlap [$F_{(1,\ 10)} = 0.23$, ns] between A-A and A-CFC rats (**Figure 5**).

FEAR-CONDITIONING-INDUCED CHANGES IN OVERLAP IN vCA3

In contrast to vCA1, vCA3 showed a significant decrease in the overlap score in the A-CFC, versus the A-A rats [$F_{(1,\ 10)} = 10.90$, $p < 0.01$; **Figures 6B** and **C**]. This decrease occurred even though the ensemble size was similar to that of the A-A group for both events (**Figure 6A**). For both groups there was a significant decrease of the ensemble size during the second event, although this decrease was not due to fear conditioning (main effect of Event: $F_{(1,\ 10)} = 34.00$, $p < 0.001$; no effect of Group or Group × Event interaction). However, fear conditioning disrupted a strong correlation between the ensemble size in dCA1 and vCA3 that emerged during exploration: there was a strong correlation between ensemble size in dCA1 and vCA3 in the A-A group ($b = +1.43$, $R^2 = 0.82$, $p < 0.01$) and no correlation in the A-CFC group ($b = -0.58$, $R^2 = 0.03$). The ensemble size in vCA3 was not correlated with the ensemble size of vCA1 for either behavioral group ($R^2 < 0.20$).

DISCUSSION

The research presented here tested two main hypotheses: (1) that conditioning fear to a place induces plasticity-related gene expression in the rodent hippocampus that is qualitatively different than that evoked by exploring the same place; and (2) that fear-conditioning-induced changes will occur preferentially in the temporal/ventral, but not septal/dorsal hippocampus. The results are congruent with the former, but not the latter hypothesis: different ensembles of cells expressing plasticity-related IEGs were observed when rats explored a place vs. when they received fear conditioning in that same place. These differences were detectable along the septo-temporal axis of the hippocampus, with the most notable effect of fear conditioning seen in the dorsal CA1 and ventral CA3. Specifically: (1) in dCA1, there was both a CFC-related decrease of ensemble size and overlap between IEG-expressing ensembles from both events in A-CFC, compared to A-A rats; (2) in dCA3, there was only a decrease in the ensemble size during the CFC and a statistically non-significant decrease in overlap for the A-CFC rats; (3) in vCA1, neither differences in ensemble size nor overlap were noted; (4) in vCA3, A-CFC rats

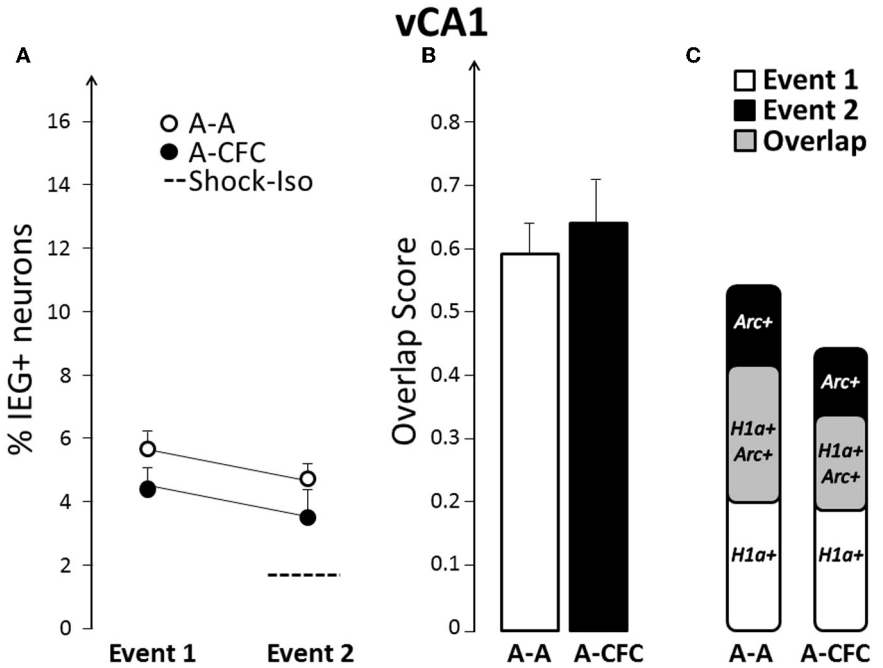

FIGURE 5 | Comparison of ensemble size and overlap between vCA1 ensembles activated during both events in A-A and A-CFC groups: (A) Size of IEG-expressing ensembles expressed as percentage of total cells. The dashed line is the percentage of vCA1 cells expressing *Arc* in response to foot shock alone. **(B)** Overlap scores; **(C)** Venn diagrams representing both size and overlap.

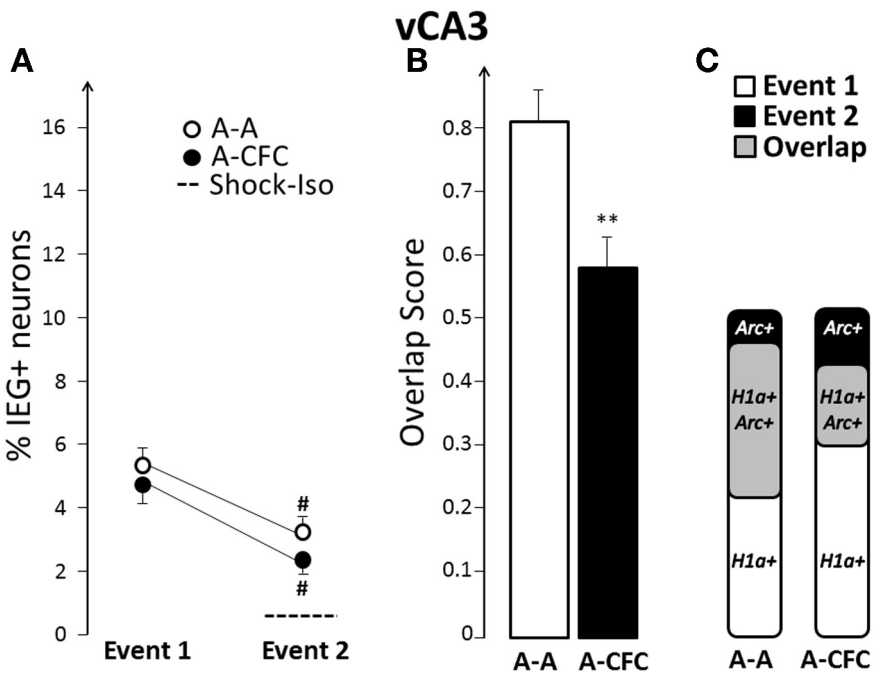

FIGURE 6 | Comparison of ensemble size and overlap between vCA3 ensembles activated during both events in A-A and A-CFC groups: (A) Size of IEG-expressing ensembles expressed as percentage of total cells. The dashed line is the percentage of vCA3 cells expressing *Arc* in response to foot shock alone. **(B)** Overlap scores; **(C)** Venn diagrams representing both size and overlap. $^{**}p < 0.01$ compared to the A-A group; $\#p < 0.05$ compared to Event 1 of the same group.

had a significant decrease in overlap compared to A-A rats. Thus, an emotionally aversive event is represented with a pattern of plasticity-related gene expression along the septo-temporal axis of the hippocampus that is distinct from that elicited by spatial exploration alone.

CONSIDERATIONS OF NOMENCLATURE

While there has been little ambiguity as to the anatomical location of the term dorsal/septal hippocampus, what has been referred to as ventral/temporal hippocampus varies significantly in the literature, depending on how many divisions are included and what is used as relative location markers. Additionally, recent accounts have identified dorsal, intermediate, ventral and ventral-ventral divisions based on a combination of molecular markers (reviewed in Fanselow and Dong, 2010). Our choice of imaging fields from the ventral part of the hippocampus (**Figure 2**) was based on the projection patterns of the BLA (Pikkarainen et al., 1999; Petrovich et al., 2001), which cover the border of what would be considered intermediate and ventral hippocampus, based on a classification derived from a combined location/molecular markers perspective (Thompson et al., 2008; Dong et al., 2009). The BLA is involved in emotional memory and influences hippocampal plasticity in the dorsal hippocampus (McGaugh, 2004; Nakao et al., 2004). This likely occurs indirectly through projections to the lateral entorhinal cortex (Pikkarainen and Pitkänen, 2001), suggesting that direct BLA projections to the more ventral parts of the hippocampus could influence encoding of emotional events by that region. Experiments that are testing this hypothesis are currently underway. The ventral regions that we analyzed also correspond to those in which protein synthesis is necessary for proper consolidation of fear conditioning (Rudy and Matus-Amat, 2005).

IEG EXPRESSION IS NOT A MEASURE OF LOCOMOTION

One of the main observations in the study is that there is a notable decrease in the size of IEG+ neuronal ensemble in several hippocampal regions during CFC. One could argue that this decrease in the size of IEG+ ensembles results from the decreased locomotion observed in the A-CFC group, compared to the control group that explored the apparatus twice without foot shock (A-A group). However, there was no correlation between the number of crossings and *Arc/H1a* expression, showing that suppressed locomotion cannot account for the decrease in IEG ensemble size. This is consistent with an increasing body of evidence which suggests that *Arc* and *H1a* are markers of plasticity and not neuronal activity, *per se* (Kelly and Deadwyler, 2003; Vazdarjanova and Guzowski, 2004; Guzowski et al., 2006; Carpenter-Hyland et al., 2010; McCurry et al., 2010). Therefore, our findings suggest that CFC reduces the number of dCA1 and dCA3 neurons undergoing plasticity during the aversive experience and does not simply reflect decreased locomotion. The decreased ensemble size may result from any of a number of reasons, such as suppressed plasticity in the hippocampus due to elevated levels of stress hormones during CFC, sped-up consolidation, or some other emotion-influenced process. Suppressed plasticity is an unlikely explanation, because stress hormones released from the adrenals in response to a stressor either enhance Arc expression: norepinephrine in the BLA, in concentrations that enhance memory, increases hippocampal Arc (McIntyre et al., 2005); or, as is the case with glucocorticoid levels in the hippocampus, are significantly elevated above baseline at 30 min (Thoeringer et al., 2007), which is much longer than 2 min—the time period required to influence *Arc* expression. Sped-up consolidation is a plausible explanation because in the course of spatial memory consolidation the size of *Arc*+ neuronal ensembles in the hippocampus and parietal cortex activated during learning shrinks in half (Ramírez-Amaya et al., 2005). Additionally, when memories are well-consolidated, the size of *Arc*+ neuronal ensembles is significantly smaller than immediately after memory acquisition (Carpenter-Hyland et al., 2010).

The lack of correlation between *Arc/H1a* expression and locomotion is consistent with previous findings showing that Arc is a marker of long-term plasticity and memory and not a marker of acquisition; Arc protein is involved in memory consolidation and not acquisition (Guzowski et al., 2000; Plath et al., 2006; Wang et al., 2006; Ploski et al., 2008; Holloway and McIntyre, 2011). Therefore, our findings should be interpreted as affecting long-term memory processes and not memory acquisition.

THE DORSAL HIPPOCAMPUS ENGAGES DIFFERENT IEG+ NEURONAL ENSEMBLES WHEN A SPATIAL CONTEXT IS EXPLORED ALONE AND WHEN IT IS ASSOCIATED WITH AN EMOTIONAL EVENT

The fear conditioning training induced robust freezing, both immediately and 24 h after training, showing that, in the A-CFC but not the A-A group, the spatial context was associated with the fear-eliciting stimulus, foot shock. The CFC training also decreased the relative proportion of IEG-expressing cells in both the dCA1 and dCA3 that would normally re-express *Arc* and *H1a* when a novel place is visited for a second time, as illustrated on **Figures 3C** and **4C**. The differences in ensemble size and overlap between the A-A and A-CFC groups are likely due to differences in *what* the two groups experienced during the second event: exploring the context vs. associating it with foot shock, because the "where" and "when" parts of their experiences were matched. It is important to note that experiencing foot shock alone, without associating it with the context, could not explain the findings. Administering a shock without giving a chance for the rats to form a representation of the spatial context, a.k.a. "immediate shock" training, is unable to support CFC memory (Fanselow, 1980). We found no evidence that administering such immediate shock induces any significant IEG expression. Even if footshock induced *Arc* expression in the dorsal hippocampus, it should lead to a larger ensemble size during CFC, not smaller, as our data showed. Previous findings of increased IEG expression in an immediate shock group (Lonergan et al., 2010) may be related to the subsequent learning that occurred ("I got shocked, although I don't know exactly where") as evidenced by enhanced exploratory and burrowing behavior that occurs when rats are returned to their home cages after immediate footshock. To prevent this additional learning, we anesthetized the immediate shock rats immediately after receiving foot shock. Such treatment does not stop already initiated transcription, but prevents initiation of transcription under anesthesia (unpublished observations from our laboratory).

These findings suggest that the dorsal hippocampus is involved in encoding emotional memories, in addition to encoding other types of episodic memory, and advance the idea that it is part of a larger memory system of brain regions that includes the basolateral and central amygdala, and the rostral anterior cingulate cortex (Maren, 2003; McGaugh, 2004; Rodrigues et al., 2004; Malin and McGaugh, 2006; Matus-Amat et al., 2007; Walker and Davis, 2008; Holloway and McIntyre, 2011). The findings also extend previous research showing that the dorsal hippocampus is necessary for single trial learning (Morris, 2001; Kesner, 2007), including single trial contextual fear conditioning (Wiltgen et al., 2006), by showing that the dorsal hippocampus creates qualitatively different representations of a context that is experienced with and without a fear-eliciting stimulus.

Although both dCA1 and dCA3 showed conditioning-induced changes, dCA1 also had a lower overlap in the A-A condition (**Figures 3B** and **4B**). This observation can reflect a hypothesized role of dCA1 in encoding the temporal and sequence dimensions of episodic memories (Hunsaker et al., 2008; Farovik et al., 2010), as the two explorations of the same place (A-A condition) differ mostly along the temporal domain. This interpretation also can explain why in the present study we observed a smaller overlap in the A-A rats than what we have observed in the past: the temporal difference between the two events may have exerted a relatively larger influence than the spatial cues which were fewer than those used in previous investigations (Vazdarjanova et al., 2002; Vazdarjanova and Guzowski, 2004; Ramírez-Amaya et al., 2005). The fact that dCA3, compared to dCA1, had a less pronounced decrease in overlap in the A-CFC rats can reflect the computational characteristics of dCA3 in respect to pattern completion and pattern separation which have been evoked to explain differences in electrophysiological activity and IEG expression during spatial learning (Lee et al., 2004; Leutgeb et al., 2004; Vazdarjanova and Guzowski, 2004; Rolls and Kesner, 2006).

These findings were made possible because of the power of within animal experimental design combined with the temporal resolution of *Arc* and *H1a* expression visualized using the *Arc/H1a* catFISH method. Simply measuring the mRNA or protein levels of IEGs or other plasticity-related molecules would not have revealed the differences between the A-A and A-CFC groups, because the combined percentage of neurons with IEG expression from both events is similar in both groups. Indeed, no difference was reported for Arc protein levels in the dorsal hippocampus between a group that explored a place and a group that received fear conditioning in that place (Lonergan et al., 2010). However, we report striking differences in overlap showing that, at a network level, the same place is encoded by demonstrably different neuronal ensembles when it is experienced alone vs. when it is associated with an aversive event.

DIFFERENCES IN FEAR-CONDITIONING-INDUCED IEG EXPRESSION ALONG THE SEPTOTEMPORAL AXIS OF THE HIPPOCAMPUS

Evidence of functional dissociations along the septotemporal axis of the hippocampus for spatial tasks has long been known (Hock and Bunsey, 1998; Moser and Moser, 1998; Hunsaker et al., 2008; Bast et al., 2009). *Arc* expression patterns during retrieval of spatial memory have also been documented (Gusev et al., 2005). Furthermore, lesions, blocking NMDA receptors, or blocking protein synthesis in the ventral hippocampus all impair long-term, but not short-term, memory for fear conditioning, while such manipulations of the dorsal hippocampus have less consistent effects (Zhang et al., 2001; Maren and Holt, 2004; Rudy and Matus-Amat, 2005; Rogers et al., 2006; Yoon and Otto, 2007). Evidence that ventral hippocampal lesions impair fear and anxiety behaviors (Kjelstrup et al., 2002; Blanchard et al., 2005), and evidence that amygdala-hippocampal projections terminate heavily in this region, led to the hypothesis that fear-conditioning-induced changes in IEG expression will be more notable in the ventral, compared to the dorsal hippocampus. Our data does not support this hypothesis: vCA1 did not show fear-conditioning-induced changes, while vCA3 did show changes, along with dCA1 and dCA3. The data suggest that both dorsal hippocampus and vCA3 may participate in encoding into long-term memory fear-conditioning-induced associations, in addition to encoding other information, i.e., spatial and temporal information, because the changes in A-CFC rats are beyond the changes observed in A-A rats. This interpretation is consistent with findings that ventral/intermediate hippocampus is necessary for spatial memory (Ferbinteanu et al., 2003; Rudy and Matus-Amat, 2005), as well as long-term memory of contextual fear (Hunsaker and Kesner, 2008), and suggests that ventral hippocampus, like dorsal hippocampus, carries out associative functions for ongoing events, including spatial and emotional aspects, when present.

In summary, contextual fear conditioning, but not experiencing foot shock alone, produced a distinct pattern of long-term plasticity-related IEG expression along the septo-temporal axis of the hippocampus when compared to spatial learning in the same place. We propose that these differences reflect the acquisition of stimulus-stimulus associations, during fear conditioning, by the rodent hippocampus, in accordance with observations made in humans.

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Enhanced emotional reactivity after selective REM sleep deprivation in humans: an fMRI study

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Converging evidence from animal and human studies suggest that rapid eye movement (REM) sleep modulates emotional processing. The aim of the present study was to explore the effects of selective REM sleep deprivation (REM-D) on emotional responses to threatening visual stimuli and their brain correlates using functional magnetic resonance imaging (fMRI). Twenty healthy subjects were randomly assigned to two groups: selective REM-D, by awakening them at each REM sleep onset, or non-rapid eye movement sleep interruptions (NREM-I) as control for potential non-specific effects of awakenings and lack of sleep. In a within-subject design, a visual emotional reactivity task was performed in the scanner before and 24 h after sleep manipulation. Behaviorally, emotional reactivity was enhanced relative to baseline (BL) in the REM deprived group only. In terms of fMRI signal, there was, as expected, an overall decrease in activity in the NREM-I group when subjects performed the task the second time, particularly in regions involved in emotional processing, such as occipital and temporal areas, as well as in the ventrolateral prefrontal cortex, involved in top-down emotion regulation. In contrast, activity in these areas remained the same level or even increased in the REM-D group, compared to their BL level. Taken together, these results suggest that lack of REM sleep in humans is associated with enhanced emotional reactivity, both at behavioral and neural levels, and thus highlight the specific role of REM sleep in regulating the neural substrates for emotional responsiveness.

Keywords: REM sleep deprivation, emotional reactivity, emotion, fMRI

INTRODUCTION

Several studies have recently investigated whether sleep plays a role in emotional regulation. These studies have shown a negative impact of total sleep deprivation (TSD) on mood and emotional processing (Dinges et al., 1997; Zohar et al., 2005; Franzen et al., 2008). For example, following TSD subjective responses to frustration and outward expressions of negative responses are augmented (Kahn-Green et al., 2006), emotional intelligence and coping skills are decreased (Killgore et al., 2008), the ability to make subtle discriminations of complex emotional blended face expressions is adversely affected (Huck et al., 2008), decision-making under uncertainty is compromised (Killgore et al., 2006; McKenna et al., 2007) and utilitarian judgments are favored (Killgore et al., 2007). Furthermore, sleep loss affects the neural systems associated with risky decisions (Venkatraman et al., 2007) and with successful recognition of emotional material (Sterpenich et al., 2007). In particular, larger and more extended amygdala activation during viewing increasingly negative stimuli has been found after TSD (Yoo et al., 2007). Taken together, these studies provide strong support to the notion that sleep is necessary for normal emotional functioning. However, as all these studies used TSD, they did not address the important question of whether all phases of sleep are critical for emotional processing or whether, for instance, non-rapid eye movement (NREM) and rapid eye movement (REM) sleep play different roles in emotion regulation.

Indeed, sleep neurobiology suggests that REM sleep may be particularly related to emotion. Emotional brain systems are selectively activated during this phase of sleep relative to wakefulness, including the amygdaloid complex, anterior cingulate, medial and orbital frontal cortices, and posterior association areas (Maquet et al., 1996; Braun et al., 1997). Magnetoencephalography current density is increased several milliseconds prior to REM sleep rapid eye movements in right amygdala and parahippocampal gyrus (Ioannides et al., 2004). Behaviorally, enhanced negative ratings (Wagner et al., 2002; Gujar et al., 2011) and memory for emotional stimuli following periods of sleep rich in REM (Wagner et al., 2001; Nishida et al., 2009) have been reported.

Studies in experimental animals have directly shown that REM sleep deprivation (REM-D) has a significant effect on emotion: shock-induced fighting is augmented (Morden et al., 1968) and fear is diminished (Hicks and Moore, 1979; Martínez-González et al., 2004). These findings suggest that REM sleep contributes to reestablish the adequate emotion-related neural excitability necessary on the following day for evaluation of potential danger and

for controlling reactivity to emotional, particularly threat-related stimuli (Horne, 2000; Walker, 2009; Walker and van der Helm, 2009), and that when this is not achieved, emotional reactivity to such cues is enhanced.

Although several behavioral and neuroimaging studies have been carried out concerning the role of REM sleep in memory processes in humans (for review: Rauchs et al., 2005; Walker and Stickgold, 2006; Chuah and Chee, 2008), very few studies have directly investigated the role of selective REM-D on emotional regulation. Early studies found that, consistent with animal studies, subjects deprived of REM sleep showed irritability and anxiety (Dement and Fisher, 1963), deterioration in interpersonal relationships and increased signs of confusion and suspicion (Agnew et al., 1967), as well as poorer adaptation to stress-induced by viewing an emotionally arousing film (Greenberg et al., 1972). However, the consequences of REM-D on reactivity to threat-related stimuli and, in particular, its cerebral correlates have not yet been investigated in humans.

Therefore, the aim of the present study was to explore the effect of selective REM-D on emotional reactivity to threatening visual stimuli and its brain correlates using functional magnetic resonance imaging (fMRI). Given that it has been proposed that REM sleep plays a role in reprocessing emotional information (Wagner et al., 2001, 2002; Nishida et al., 2009) and in stabilizing emotional system reactivity (Horne, 2000; Walker, 2009; Walker and van der Helm, 2009), a within-subject design was employed in which subjects were scanned twice, before and after one night of either selective REM-D or NREM sleep interruptions (NREM-I). In both tests, the same paradigm and stimuli was used in order to better allow for a direct comparison between conditions.

MATERIALS AND METHODS

SUBJECTS

Twenty right-handed adult male volunteers between 21 and 35 years of age (average 24.2; SD 4.0) participated in the fMRI study after giving their written informed consent. They were recruited from the university community (mean education = 16.5; SD = 1.4 years) and received financial compensation for their participation. Before the experiment, subjects completed a structured clinical interview and kept a sleep log of 15 days to check for the absence of sleep disturbances and to record their usual bedtime and sleep duration. Only volunteers with regular sleeping habits (average 7.38 h/night; SD 0.09), and regular bedtime hours (from 22:00–24:00 h to 06:00–08:00 h), with no symptoms associated with sleep disorders, no history of medical, psychiatric or neurological disorders, and free of drugs or medications were included in the study. All participants scored below the cutoff point for moderate depression on the Beck Depression Inventory (average 3.6; SD 3.2). All subjects had normal or corrected-to-normal vision. The protocol was approved by the Ethical Committee of the Faculty of Medicine of the National Autonomous University of Mexico.

Subjects were randomized into two groups: One group was selectively REM-sleep deprived ($n = 12$) and the other ($n = 8$) was submitted to the same number and distribution across the night of NREM-I to control for non-specific effects of sleep disruption and fragmentation. Due to excessive movement during scanning, data from one participant in the NREM-I group had to be discarded.

SLEEP MANIPULATION

All participants slept for four consecutive nights at the laboratory in a soundproof room with controlled temperature: the first for adaptation to recording procedures, the second as baseline (BL), the third for experimental treatment (EXP)—either REM-D or NREM-I—, and the fourth night to assess sleep recovery (REC). All participants were required to abstain from caffeine-containing beverages and alcohol, and to avoid napping 3 days before the adaptation night and throughout the experiment. After the REM-D or NREM-I nights, subjects remained near the laboratory and were supervised continuously during all day to prevent naps. Breakfast, lunch, and dinner were provided.

Standard polysomnography (PSG), electroencephalography (EEG), electrooculogram (EOG), and electromyogram (EMG) were recorded during BL, experimental and recovery nights. Electrical activity was recorded using a Grass 8–20 E polygraph with filters set at 1 and 70 Hz for EEG, at 10 and 70 for EMG and at 0.3 and 15 for EOG. Sleep stages were identified according to standard procedures using 30 s epochs (Rechtschaffen and Kales, 1968). Sleep stage percentages were calculated over total sleep time (time from sleep onset to morning awakening after the time awake during the night is subtracted), and wakefulness after sleep onset (WASO) was calculated from sleep onset to morning awakening over total sleep time plus wakefulness. The latter included spontaneous awakening during the BL and REC nights, and both spontaneous and experimentally induced awakenings during the EXP night.

Subjects in the selective REM sleep deprived group were awakened every time that the PSG showed that slow wave activity, sleep spindles and K complexes, were no longer present in the EEG which, instead, was characterized by low voltage fast activity accompanied by decreased EMG activity for 60 s with or without REMs (Rosales-Lagarde et al., 2009). Participants were awakened by the experimenter entering the recording chamber with lights-off and by increasing the volume of a previously recording tape with the name of the subject until the subject was fully awake. They were kept awake during 2 min by asking them incidental questions (e.g., name the days of the week), to avoid the immediate relapse into REM sleep (Endo et al., 1998). The subjects of the NREM-I group were awakened in the same manner but from stages 2, 3, or 4 of NREM sleep.

Sleep variables were compared by Two-Way mixed ANOVAs (groups x nights) with the two groups as the between-subject factor and the three PSG nights (BL, EXP, and REC) as the within-subject factor. Significant interactions were further tested with Tukey studentized t-tests.

EXPERIMENTAL TASK

All participants performed the Emotional Reactivity Task in the MR scanner in two separate sessions, once in the evening after BL night and again in the evening after either REM-D or NREM-I. The same stimuli were used in both sessions. To avoid confounding circadian effects, scanning sessions took place at the same time of day around 6 pm (between 5 and 7 pm). Additionally, as a

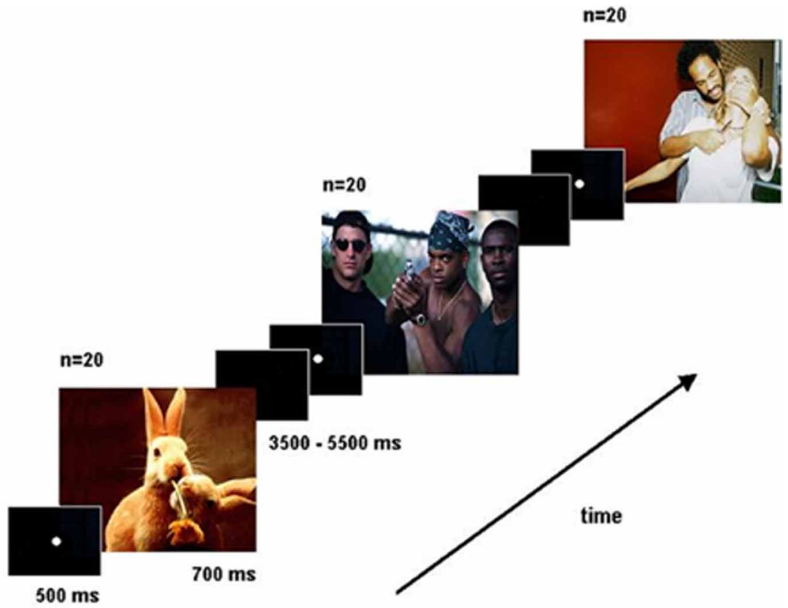

FIGURE 1 | Schematic of the Emotional Reactivity Task.

control for unspecific effects of sleep interruption on attention, all subjects also performed outside the scanner a visual sustained attention task (VISAT) that has been previously employed to assess sleep deprivation effects (Corsi-Cabrera et al., 1996).

The emotional reactivity task (**Figure 1**) involved the presentation of 60 pictures from the International Affective Pictures System (Lang et al., 2005). Subjects were instructed to imagine themselves as part of the scene and react to the situation as soon as possible either by defending themselves (i.e., firing a bullet) or not, by pressing one of two buttons with the right index or middle finger (counterbalanced across sessions and subjects). Thus, a specific response was required for every slide in order to control for motor response activations. Pictures were selected according to the normative valence ratings and were classified into two extreme valence categories, 40 negative valence pictures (20 directly threatening the observer and 20 threatening a third party, mean valence = 3.46), and 20 non-menacing positive pictures (mean valence = 7.19). Trials were assigned *a posteriori* to one of two categories based on each subject's response: those eliciting a defense response were labeled as high emotional reactivity trials (HER) and non-defending responses as low emotional reactivity trials (LER). All pictures were shown in a randomized order. Each picture was presented for 700 ms preceded by a fixation point (500 ms) at the center of the screen and followed by a black screen. The duration of the inter-trial black screen varied randomly between 3.5 and 5.5 s.

The number of HER and LER responses and reaction times were computed and analyzed using single and independent *t*-tests.

IMAGING PROCEDURE AND ANALYSIS

Functional MRI data was acquired with a General Electric 1.5 Tesla MR system at the Hospital Angeles del Pedregal. For each subject and each session, 120 echo-planar image volumes were acquired (8 6 mm-thick axial slices with the third one positioned parallel to the anterior-posterior commissure; TR = 3 s; TE = 27 ms; interslice gap: 4 mm; field of view: 24 × 24 cm, matrix: 128 × 128), in two runs of 60 scans with three dummy scans discarded before analysis due to T1 saturation effects. Before the functional runs, a T1-weighted anatomical scan was obtained (TR = 18.4 ms, TE = 4.2 ms, 0.47 × 0.47 × 3 mm^3 voxel size).

Functional data was preprocessed following standard procedures using SPM2 (www.fil.ion.ucl.ac.uk/spm/software/spm2). Briefly, functional images for each subject were realigned to the first image of each session to correct for head movement between scans and corrected for differences in slice acquisition times. Images were then spatially normalized to the Montreal Neurological Institute (MNI) space (Evans et al., 1994) and spatially smoothed using an isotropic Gaussian kernel of 8 mm Full width at half maximum (FWHM).

Events were modeled as pseudo-delta functions coinciding with stimulus onset, convolved with the synthetic hemodynamic response function. Stimuli were divided into high- and low-emotional reactivity based on each subject's response, as indicated above.

Brain responses associated with each experimental condition were estimated according to the general lineal model for an event-related design at each voxel. In order to identify the brain regions associated with the performance of the Emotional Reactivity Task, we first analyzed cerebral activity under BL sleep conditions (i.e., prior to sleep deprivation/interruption). To characterize the effects of the sleep manipulation on the Emotional Reactivity Task, contrasts were obtained comparing BL and REM deprivation nights (PRE-REM-D minus POST-REM-D), or BL and NREM interruption nights (PRE-NREM-I minus POST-NREM-I). These contrasts were computed for task related

activations (HER + LER) and for emotional reactivity (HER – LER). Statistical maps were thresholded at $p < 0.001$ (uncorrected for multiple comparisons), with a minimum cluster size of 10 voxels. In addition, parameter estimates for the activation peaks obtained in the contrasts of interest were extracted for plotting and to conduct further *post-hoc* *t*-tests. To further confirm that the differences in activation before and after sleep manipulation were indeed related to REM sleep, we performed regression analyses between the contrasts of interest and percentage of REM sleep for all 19 subjects.

RESULTS

SLEEP

There were no significant differences between groups in the mean number of hours of sleep in the 2 weeks prior to the study, as recorded from the subjects' sleep diaries (REM-D = 7.26, SD 0.87; NREM-I = 7.48, SD 1.05; $t = 0.39$, $p = 0.7$).

As shown in **Figure 2**, REM-D procedure successfully reduced REM sleep in the REM-D group only. Statistical results for ANOVAs are shown in **Table 1**. REM sleep percentage differed significantly between groups and nights. *Post-hoc* comparisons

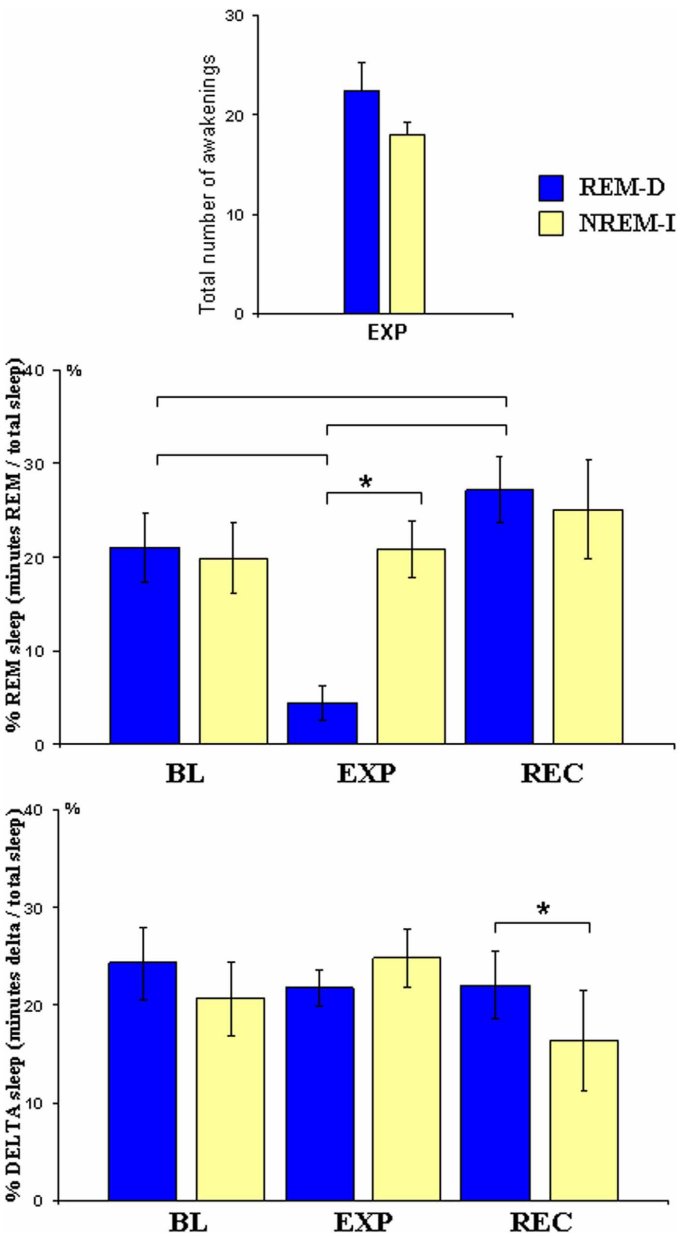

FIGURE 2 | Mean and standard error of total number of experimental awakenings during sleep manipulation night, and of percentage of REM sleep (minutes of REM/minutes total sleep) and delta sleep (minutes of stage3 + 4/min total sleep) at baseline (BL), experimental (EXP) and recovery (REC) nights for the REM sleep deprivation (REM-D), and non-REM sleep interruptions (NREM-I) groups; Brackets indicate significant *post-hoc* differences in the REM-D group whereas asterisk above bracket represents differences between groups.

Table 1 | Polysomnographic sleep measures.

	REM-D group			NREM-I group			Mixed ANOVA		
	BL	REM-D	REC	BL	NREM-I	REC	Group df = 1,17	Night df = 2,34	Interaction df = 2,34
	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	Mean (SD)	*p* (F)	*p* (F)	*p* (F)
TST (min)	446.3 (38.0)	350.3 (47.0)	447.2 (49.6)	473.9 (55.4)	426.2 (44.1)	479.6 (57.4)	0.02 (6.07)	0.0001 (26.43)	n.s.
Stage 1/TST	3.4 (1.6)	10.5 (4.5)	3.0 (1.2)	2.6 (1.8)	4.0 (1.6)	4.8 (3.2)	0.03 (5.15)	0.0001 (13.59)	0.0002 (12.15)
Stage 2/TST	51.2 (5.8)	61.9 (5.8)	47.6 (5.6)	56.9 (7.6)	50.3 (4.5)	53.8 (5.8)	n.s.	0.02 (4.10)	0.0001 (14.06)
Delta sleep/TST	24.3 (6.8)	23.5 (6.0)	22.1 (4.6)	20.7 (5.0)	24.9 (5.2)	16.4 (3.0)	n.s.	0.01 (4.83)	n.s.
REM sleep/TST	21.01 (3.7)	4.03 (1.3)	27.2 (3.5)	19.8 (3.8)	20.8 (2.9)	25.1 (5.2)	0.001 (15.66)	0.0000 (89.29)	0.0001 (53.56)
WASO/TST	2.8 (2.8)	19.7 (14.1)	3.5 (4.9)	2.2 (1.8)	14.0 (5.2)	2.9 (3.7)	n.s.	0.0001 (29.20)	n.s.
Latency to REM (min)	112.1 (31.9)	96.7 (37.7)	96.8 (35.1)	81.9 (16.7)	102.6 (24.3)	79.9 (19.7)	n.s.	n.s.	n.s.

REM-D, Rapid Eye Movement Sleep Deprivation; NREM-I, Non Rapid Eye Movement Sleep Interruptions; BL, base line night; REC, recovery night; TST, total sleep time (time from sleep onset to morning awakening after the time awake during the night is subtracted); Delta sleep refers to Stage 3 + Stage 4; REM, rapid eye movement sleep; WASO, wakefulness after sleep onset to morning awakening (TST plus wakefulness); p < 0.05.

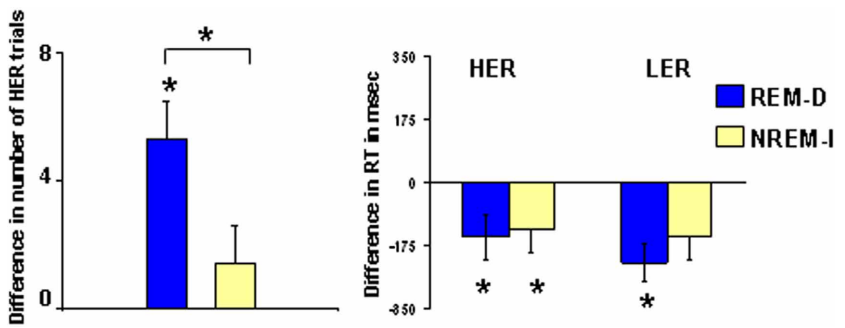

FIGURE 3 | Difference in the number of high emotional reactivity trials (HER, see Methods for details) and in reaction times for HER and low emotional reactivity trials (LER) after REM sleep deprivation (REM-D) or non-REM sleep interruptions nights (NREM-I). Asterisks above brackets indicate significant *post-hoc* differences between groups whereas asterisks above individual bars represent significant differences from zero.

showed that REM sleep percentage was significantly reduced from 21.01% over total sleep time in BL night to 4.03% during the REM deprivation night ($p < 0.01$), followed by a significant, though small, REM rebound in the recovery night ($p < 0.01$). In contrast, there were no differences in the amount of REM sleep among nights in the NREM-I group. Group-by-night interactions were also significant for Stages 1 and 2. Both sleep stages were higher for the REM-D during the experimental night. Importantly, the average number of sleep interruptions did not differ significantly between groups (**Figure 2**). The difference in standard deviation between groups was due to two subjects in the REM-D group with large number of interruptions (37 and 45). Removing these two subjects from the analyses did not substantially change the results.

Night effects were also significant for delta sleep (**Figure 2**); *post-hoc* comparisons showed that these effects were due to lower delta sleep in the recovery night ($p < 0.05$) but not during the experimental night. In addition, as it could be expected from experimental awakenings, WASO increased in both groups during the experimental night as compared to BL ($p < 0.01$) and recovery nights ($p < 0.01$), with no group differences observed. Importantly, overall sleep architecture was not modified in the NREM-I group during the experimental night and therefore this group can be used as a control group for non-specific effects due to sleep interruptions.

BEHAVIORAL RESULTS

Emotional reactivity task

As shown in **Figure 3**, the number of HER responses was increased after REM-D (before REM-D: mean = 26.3, SD = 4.8; after REM-D: mean = 31.6, SD = 4.7; $t = 4.50$; $p < 0.0009$), whereas emotional reactivity was not modified in the NREM-I group (before NREM-I: mean = 30.3, SD = 7.8; after NREM-I: mean = 31.7, SD = 2.9; $t = 1.24$; $p = 0.26$).

Reaction times decreased significantly from the first to the second time that the task was performed for HER responses in the two groups (REM-D: $t = 2.40$; $p < 0.03$; NREM-I: $t = 2.43$; $p < 0.05$), with no significant group differences. Reaction times also decreased the second time for LER responses in the REM-D group, while the difference in the NREM-I group failed to reach the standard level of significance ($t = 2.20$; $p = 0.06$).

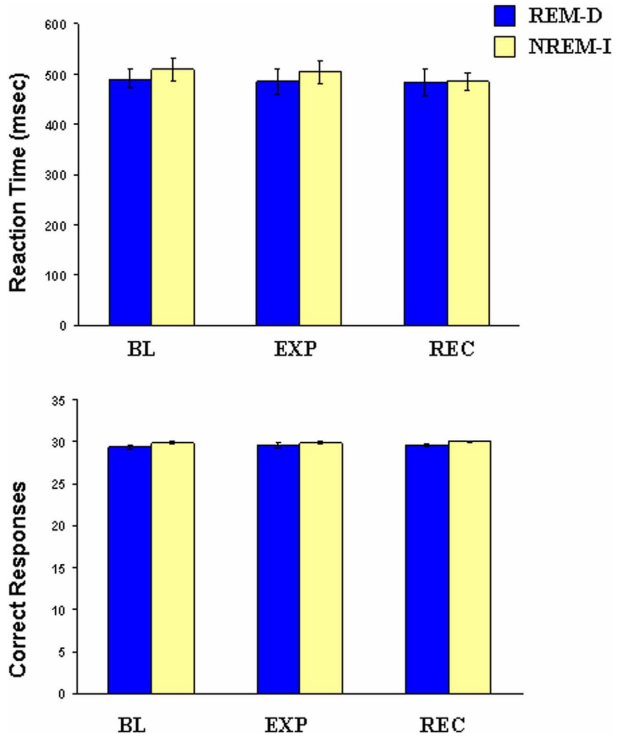

FIGURE 4 | Mean and standard error of reaction time and number of correct responses in the Visual Sustained Attention Task at baseline (BL), experimental (EXP) and recovery (REC) nights for the REM sleep deprivation (REM-D), and non-REM sleep interruptions (NREM-I) groups.

Visual sustained attention task

Reaction time and correct responses (**Figure 4**) were not significantly different in any of the two groups between sessions (reaction time: group main effect [$F_{(1,\ 17)} = 0.38$; $p = 0.55$], nights main effect [$F_{(2,\ 34)} = 0.24$; $p = 0.63$], group-by-night [$F_{(2,\ 34)} = 0.01$; $p = 0.94$]; correct responses: group main effect [$F_{(1,\ 17)} = 1.64$; $p = 0.21$], night main effect [$F_{(2,\ 34)} = 0.11$; $p = 0.74$], group-by-night [$F_{(2,\ 34)} = 0.11$; $p = 0.74$]).

FUNCTIONAL MRI RESULTS

Baseline

Table 2 shows the significant activations for the main effect of the Emotional Reactivity Task (HER + LER) after BL sleep (i.e., before sleep manipulation) in the 19 subjects. This contrast revealed large activations of right superior (BA9), middle (BA6) and inferior (BA46/9; BA45) frontal gyri, insula (BA47), (BA32/8/6), precentral cortex (BA4), inferior parietal lobule (BA2/3/40) and precuneus (BA7), and in middle and inferior frontal gyri (BA9/9/6; BA44/45/46), medial frontal gyrus (BA10), fusiform gyrus (BA19/18) and caudate tale of the left hemisphere.

The contrast of HER minus LER trials showed that high emotional responses resulted in significant increased activation in the right inferior frontal gyrus (BA45/47), while no significant activations associated with the opposite contrast (LER – HER) were observed.

Effects of sleep manipulation

Activation associated with task performance [(HER + LER) vs. fixation] decreased after NREM-I in comparison to post BL-sleep session (**Figure 5A**), as it could be expected for a second exposure to the same stimuli (**Table 3**). Significant decreased activations were seen in right inferior and middle frontal gyri (BA45; BA46 and BA11) and right fusiform gyrus (BA20), as well as in left superior frontal gyrus (BA11), inferior parietal lobule (BA40), posterior middle temporal gyrus (BA21) and parahippocampal gyrus.

In contrast, very little difference was observed when comparing the first and second sessions in the REM-D group. Significant deactivations associated with the Emotional Reactivity Task performance after REM-D, as compared to post BL-sleep (**Table 3** and **Figure 5B**), were only found in left anterior (BA32) and posterior cingulate gyrus (BA30 and BA31). Furthermore, *post-hoc* analyses of the parameters estimates for peak voxels of decreased activation in the NREM-I group revealed no significant effects for the REM-D group (p's > 0.05), except for the left posterior middle temporal gyrus (BA21) where it also decreased significantly ($p = 0.02$) in the REM-D group (**Figure 5A**).

The reverse comparison revealed that only the left thalamus showed increased activation in the NREM-I group as a function of sleep manipulation, compared to BL, without any change in the REM-D group for this region.

In terms of emotional responses, comparison of the high- and low-emotional reactivity trials (HER > LER) before and after sleep manipulation showed that HER trials elicited increased activation in the left middle occipital gyrus (BA19/18) following REM-D. As shown in **Figure 6** and **Table 3**, *post-hoc* analyses of the parameters estimates revealed that, in contrast, activity in this region decreased significantly after NREM-I. The reverse contrast did not show significant differences. The contrast between HER and LER trials in the NREM-I group did not differ significantly from BL session.

Regression analysis

Significant activations obtained in the correlations between brain activity and percentages of REM sleep for all subjects are shown in **Table 4**.

For the contrast of task vs. BL, we observed a negative correlation in left middle temporal gyrus (BA21) in the same region that was deactivated in the NREM-I group, as well as in the cerebellum bilaterally; whereas REM sleep percentage correlated positively with activation in the right putamen. For the HER > LER, a negative correlation was found with REM sleep percentage bilaterally in the cingulate gyrus (BA23), lingual gyrus (BA17/18) and fusiform gyrus (BA18 and extending to BA19 in the left hemisphere), in right precuneus and superior parietal lobule (BA7) and in left inferior frontal gyrus (BA9) and left cerebellum. Interestingly, the activations in visual areas were similar to those found to be more active after sleep manipulation in the REM-D group.

Table 2 | Activations during the emotional reactivity task after baseline sleep.

Brain region	BA	Coordinates			*Z* score	Cluster size
		x	*y*	*z*		
EMOTIONAL REACTIVITY TASK PERFORMANCE						
Right middle frontal gyrus	46	52	22	26	4.95	1018
Right inferior frontal gyrus	9	56	12	32	4.48	
Right middle frontal gyrus	46	56	36	16	3.46	
Right superior frontal gyrus	9	24	40	32	4.28	78
Right inferior frontal gyrus	47	32	32	−14	3.65	71
		34	24	−16	3.35	
Right inferior frontal gyrus	45	52	16	4	3.46	44
Right insula	47	32	16	0	3.76	57
Right cingulate gyrus	31	8	−32	40	3.70	254
	24	4	−20	40	3.42	
Right middle frontal gyrus	6	10	−20	48	3.38	
Right middle frontal gyrus	6	26	−6	46	3.46	57
		34	−10	42	3.39	
Right precentral gyrus	4	62	−12	36	4.05	129
Right superior parietal lobule	7	32	−58	54	3.67	85
Right precuneus	7	24	−66	36	3.59	160
	19	32	−70	34	3.46	
Right precuneus	7	28	−74	54	3.49	26
Right postcentral gyrus	2	48	−24	46	4.36	627
	3	40	−20	48	4.16	
Right inferior parietal lobule	40	42	−36	50	3.87	
Left medial frontal gyrus	10	−10	68	4	3.52	53
Left inferior frontal gyrus	9	−48	6	26	3.97	412
Left middle frontal gyrus	9	−54	16	30	3.76	
	6	−52	4	50	3.4	
Left precentral gyrus	44	−58	16	6	3.82	219
Left inferior frontal gyrus	45	−56	28	6	3.46	
Left middle frontal gyrus	46	−48	32	18	3.43	
Left inferior frontal gyrus	47	−42	28	−18	4.59	264
Left medial frontal gyrus	8	0	20	46	3.94	656
	6	−6	2	48	3.93	
Right cingulate gyrus	32	8	20	42	3.77	
Left middle frontal gyrus	6	−38	2	46	3.66	62
Left fusiform gyrus	19	−42	−72	−14	6.09	17286
Left inferior occipital gyrus	18	−38	−82	−12	5.84	
Right declive of cerebellum		34	−56	−14	5.84	
Left caudate tale		−34	−32	0	4.07	53
HER > LER TRIALS						
Right inferior frontal gyrus	45	50	22	10	4.02	225
	45	54	28	4	3.84	
	47	48	30	−6	3.19	

All of the regions presented are significant at $p < 0.001$ (uncorrected).

BA, Brodmann area; HER, high emotional reactivity trials; LER, low emotional reactivity trials (see Materials and Methods for details).

DISCUSSION

In the present study we examined the impact of selective REM-D on emotional reactivity to threatening visual stimuli and the associated brain activity changes. The main findings are that emotional reactivity, as assessed by defensive responses (HER trials), was enhanced after one night of near-total REM-D, while reactivity did not change after an equal number of NREM-I. Neural systems associated with task performance were differently affected by REM-D than by NREM-I.

Importantly, the observed effects are likely to be specifically due to REM-D, given that the procedure successfully reduced REM sleep to only 4% over total sleep time in the REM-D

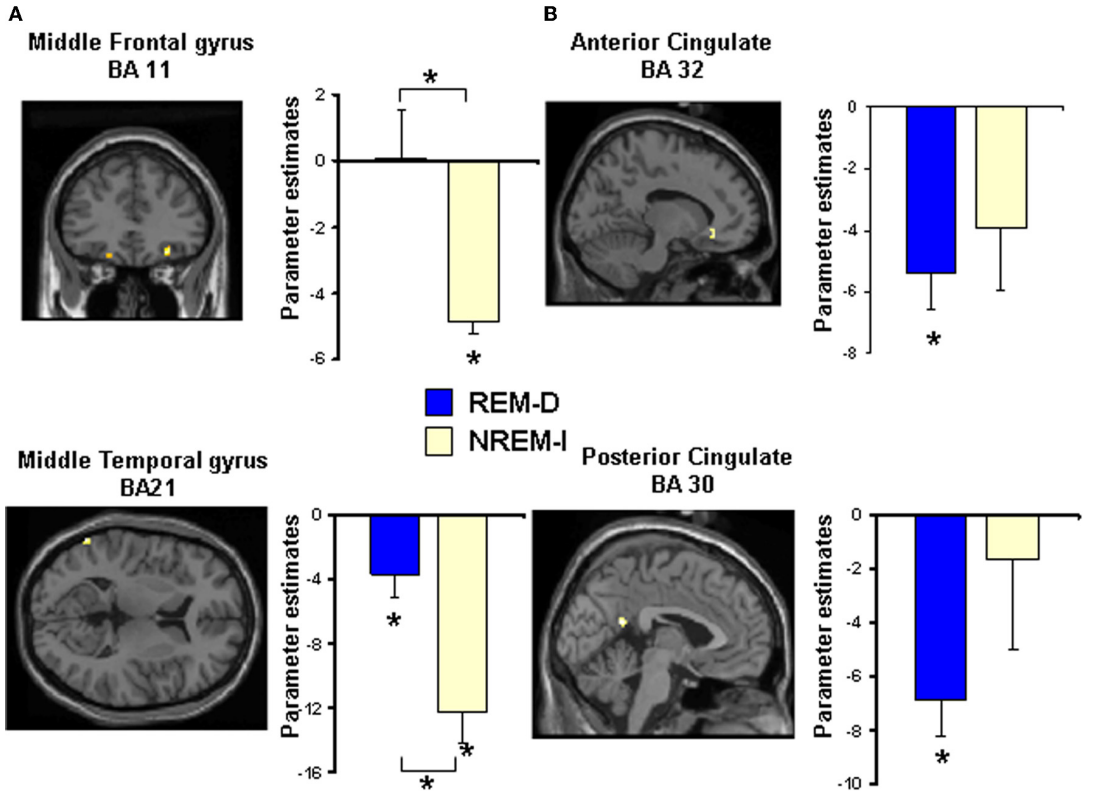

FIGURE 5 | Statistical maps for some of the regions showing significant differences in activation during performance of the Emotional Reactivity Task (high + low emotional reactivity trials) after (A) non-REM sleep interruptions (NREM-I) and (B) REM sleep deprivation (REM-D), relative to baseline sleep. Maps are thresholded at $p < 0.001$ (uncorrected). See **Table 3** for all significant activations. Bar graphs show the corresponding differences in activation between baseline and experimental night for the REM sleep deprivation and non-REM sleep interruption groups. Coordinates are in MNI space. Asterisks above brackets indicate significant *post-hoc* differences between groups whereas asterisks above individual bars represent significant differences from zero ($p < 0.05$).

deprivation night without altering delta sleep during the experimental night. Furthermore, the enhanced emotional reactivity after REM-D cannot be attributed to non-specific effects on attention, given that the VISAT did not differ in any of the two groups after sleep manipulation.

The Emotional Reactivity Task under BL sleep conditions activated areas that have been previously shown to be implicated in emotional response selection (Mitchell, 2011), including prefrontal regions and temporal areas. Critically, all of these cortical regions, except for the cingulate gyrus, decreased their activation after NREM-I, as it could be expected for a second exposure to the same task. Although the aim of the study was not to directly test memory, the Emotional Reactivity Task was performed twice, before and after sleep manipulation, and thus it is likely that the observed faster reaction times and decreased brain activation the second time that the task was performed were due to memory and/or practice effects (Chein and Schneider, 2005).

In stark contrast, in subjects who underwent selective and near-total REM-D activation remained at the same level the second time they performed the task, only significantly decreasing along the cingulate gyrus. In particular, the ventrolateral prefrontal cortex (BA45 and 47), involved in top-down emotional regulation (Mitchell, 2011), was reactivated in HER compared to LER trials at the same level as the first time they performed the task in the REM-D subjects, whereas activity in this region decreased in the NREM-I group. These results indicate that the same level of brain activation was required for appropriately performing the task a second time in the absence of REM sleep, suggesting that with insufficient REM sleep, performing the task a second time still required some degree of supervisory control from the ventrolateral prefrontal cortex, a region involved in top-down emotional regulation (Mitchell, 2011). These results could be interpreted as suggesting either a compensatory process necessary to cope with response selection, similar to what has been shown for cognitive tasks after TSD (Drummond et al., 2006), or that the lack of sufficient REM sleep impairs the reorganization and flow of information to other brain regions as it has been shown for memory processes (Buzsáki, 1989; Fischer et al., 2005).

Increased activation for HER compared to LER trials was seen after REM-D, relative to their own BL sleep, in left occipital association regions commonly activated when assigning meaning to visual emotional stimuli and in processes evaluating the affective valence of sensory events (Vuilleumier et al., 2001). In addition, increased bilateral activation in occipital and temporal visual areas showed a significant negative correlation with percentage of REM sleep in all subjects. The occipital cortex

Table 3 | Activations during the emotional reactivity task after sleep manipulation.

Brain region	BA	Coordinates			*Z* Score	REM-D Paired *t*-test		NREM-I Paired *t*-test		Independent *t*-test	
		x	*y*	*z*		*t*	*p*	*t*	*p*	*t*	*p*
EMOTIONAL REACTIVITY TASK PERFORMANCE (HER + LER)											
NREM-I Group											
Right inferior frontal gyrus	45	54	26	22	4.25	0.91	0.37	13.41	0.0001	0.89	0.38
Right middle frontal gyrus	46	46	22	20	3.88						
Right middle frontal gyrus	11	28	34	−16	4.15	0.05	0.95	12.81	0.0001	2.48	0.02
Right fusiform gyrus	20	42	−36	−14	3.78	0.87	0.40	9.81	0.0001	1.29	0.21
Left superior frontal gyrus	11	−24	40	−22	3.93	0.63	0.53	9.15	0.0001	1.32	0.20
Left inferior parietal lobule	40	−40	−54	38	4.02	0.44	0.66	14.75	0.0001	0.55	0.58
Left middle temporal gyrus	21	−62	−58	8	3.56	2.53	0.02	6.39	0.0006	3.51	0.002
Left parahippocampal gyrus		−32	−20	−12	3.26	0.21	0.83	7.77	0.0002	1.87	0.07
Left thalamus		−10	−16	10	3.56	0.009	0.99	9.81	0.0001	1.57	0.13
REM-D Group											
Anterior cingulate gyrus	32	−12	30	−10	3.38	4.61	0.0007	1.97	0.09	0.68	0.50
Posterior cingulate gyrus	30	−2	−50	20	3.64	5.13	0.003	0.50	0.63	1.70	0.10
Cingulate gyrus	31	−24	−38	38	3.58	4.74	0.0006	1.31	0.23	0.03	0.97
HIGH VS. LOW REACTIVITY TRIALS (HER > LER)											
REM-D Group											
Middle occipital gyrus	19	−30	−88	8	3.71	5.40	0.0002	0.28	1.17	3.79	0.001
	18	−18	−102	8	3.65						
	18	−26	−96	8	3.43						

All of the regions presented are significant at p < 0.001 (uncorrected).

BA, Brodmann Area; REM-D, REM sleep deprived group; NREM-I, NREM sleep interruptions group, HER, high emotional reactivity trials; LER, low emotional reactivity trials.

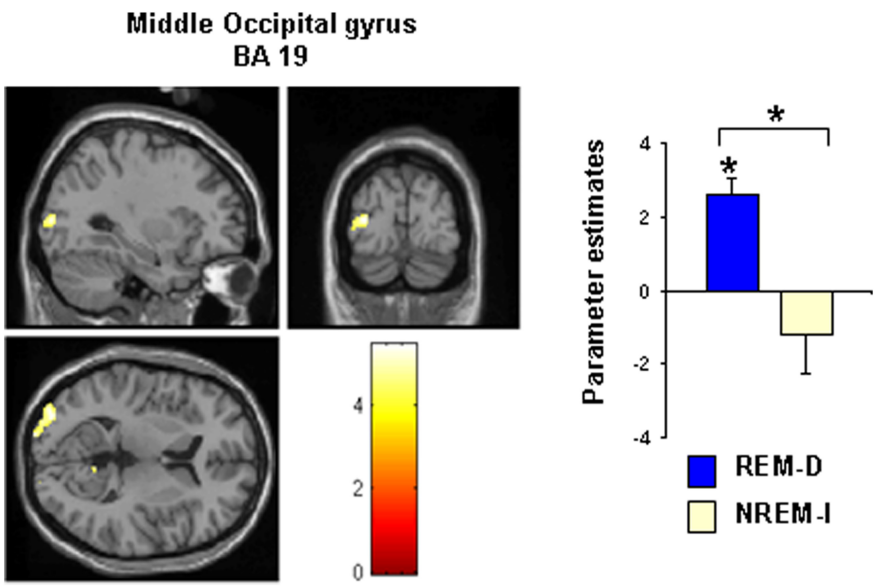

FIGURE 6 | Statistical map showing the significant activation cluster (–30 –88 8) for the contrast high reactivity trials (HER) minus low reactivity trials (LER) after REM sleep deprivation (REM-D). The map is thresholded at $p < 0.001$ (uncorrected). See **Table 3** for all significant activations. Bar graphs show the corresponding differences in activation between baseline and experimental night for the REM sleep deprivation and non-REM sleep interruption groups. Asterisks above bars and brackets indicate significant difference from zero and between groups, respectively ($p < 0.05$).

Table 4 | Result from the correlation analysis with percentage REM sleep.

Brain region	BA	Coordinates			*Z* score	Cluster size
		x	*y*	*z*		
EMOTIONAL REACTIVITY TASK PERFORMANCE						
Positive Correlation						
Right putamen		24	−12	14	3.96	56
Negative Correlation						
Right cerebellum						
Anterior lobe		18	−44	−30	4.02	15
Posterior lobe		20	−62	−28	3.31	10
Left middle temporal gyrus	21	−62	−58	8	3.59	13
Left cerebellum anterior lobe		−14	−44	−28	3.89	18
HER > LER TRIALS						
Negative Correlation						
Right precuneus	7	26	−76	54	4.09	151
Right superior parietal lobule	7	44	−68	56	3.88	
Right lingual gyrus	17	12	−98	−16	4.16	32
Right lingual gyrus	18	10	−52	4	3.22	14
Right cingulate gyrus	23		−28	28	3.85	76
Left cingulate gyrus	23	−6	−28	28	3.56	
Left lingual gyrus	17	−20	−102	−12	3.81	100
	18	−22	−96	−2	3.44	
Left cuneus	18	−16	−104	10	3.99	22
Left middle occipital gyrus	19	−54	−70	−10	3.83	25
Left declive of vermis		0	−74	−10	3.77	21

All of the regions presented are significant at $p < 0.001$ (uncorrected).

BA, Brodmann area; HER, high emotional reactivity trials; LER, low emotional reactivity trials (see Materials and Methods for details).

is one of the brain regions more strongly activated by Ponto-Geniculate-Occipital waves (PGO) during REM sleep in cats. PGO waves reach their highest amplitude in the lateral geniculate body, primary and visual association areas in cats (Datta, 1997). Although PGO waves cannot be recorded in humans, PGO-like field potentials have been recorded in humans (Lim et al., 2007), and increased activity in both lateral geniculate body and occipital cortex has been reported with Positron Emission Tomography (PET) in humans (Hong et al., 1995; Peigneux et al., 2001). It has been shown that REM-D in rats increases the activity of Na-K ATPase enzyme (Gulyani and Mallick, 1993), leading to increased excitability of neurons (Das and Mallick, 2008). The usual inhibitory period observed after repetitive auditory stimulation is shortened after REM-D in evoked potentials (Dewson et al., 1967) and in pontine neurons (Mallick et al., 1991) in cats suggesting maintained excitability to the same stimulation. The increased activation in visual areas in the REM-D suggests that the lack of incoming phasic, PGO-like, activation in the REM-D group might facilitate visual cortex excitability in response to visual stimuli.

The present findings extend previous results showing that sleep plays a crucial role in emotional regulation (Dinges et al., 1997; Zohar et al., 2005; Kahn-Green et al., 2006; Killgore et al., 2006, 2007, 2008; McKenna et al., 2007; Franzen et al., 2008) and are consistent with studies showing modification of brain networks involved in emotional processing after TSD (Sterpenich et al., 2007; Venkatraman et al., 2007; Yoo et al., 2007). Present results highlight the crucial role of REM sleep in emotion, in line with the proposed role for REM sleep for stabilizing emotional system reactivity (Horne, 2000; Lara-Carrasco et al., 2009; Walker, 2009; Walker and van der Helm, 2009; Gujar et al., 2011). In addition, our findings are in line with animal studies showing enhanced reactivity to emotional stimuli after REM-D (Morden et al., 1968; Hicks and Moore, 1979; Martínez-González et al., 2004).

Several studies suggest a role of sleep in general (Wagner et al., 2001; Hu et al., 2006; Sterpenich et al., 2007; Payne et al., 2008; Gujar et al., 2011), and REM sleep in particular, in facilitating memory for emotionally salient stimuli in humans (Wagner et al., 2001; Nishida et al., 2009). Present results may seem incompatible with previous reports suggesting that REM sleep enhances aversive reactivity to (Wagner et al., 2002), and memory for, emotional, particularly negative, stimuli (Wagner et al., 2001; Nishida et al., 2009). However, several key methodological differences between our and these previous studies should be considered. First, Wagner et al. (2002) assessed emotional reactivity by subjective evaluation, which may not be directly comparable to the motor defensive response used here. Second, in previous studies REM sleep was not directly manipulated, but, rather, it was shown that the amount of natural REM sleep, either during the second half of the night (Wagner et al., 2001) or in naps (Nishida et al., 2009) was associated with memory enhancement. Finally, in

the present study we did not directly test retention of emotional material but instead assessed reactivity by exposure to the same stimuli for a second time, which leaves open the possibility of poorer memory retention by lack of REM sleep. However, we do not believe that this is the case because although reaction times decreased in both groups for HER responses, as it could be expected for a second exposure to the same stimuli, BOLD signal increased or remained the same after REM-D instead of decreasing. The present results are consistent with the model proposed by Walker and van der Helm (2009) proposing that REM sleep might facilitate episodic memory and at the same time depotentiate the emotional tone or affective charge of the stimuli.

Taken together, these findings could be tentatively interpreted as suggesting that REM sleep is involved not only in memory enhancement but also in preparing the brain for the next day's demands, as it proposed by Walker (2009), by reprocessing information related to emotional experiences in a safe environment provided by atonia and free from high order control (Maquet et al., 1996; Corsi-Cabrera et al., 2003), and by modulating brain excitability (Das and Mallick, 2008) in a similar way as it has been proposed for sleep in general (Tononi and Cirelli, 2003). The activation of emotional systems during REM sleep may facilitate dissipation of accumulated excitability reestablishing an adequate level of emotional responsiveness for the next period of wakefulness (Corsi-Cabrera, 1983; Walker and van der Helm, 2009). In this case, the lack of REM sleep may affect the fine-tuning of networks involved in emotional reactivity by enhancing excitability and/or impairing assimilation of previous experiences, leading to hyper-reactivity and increased appraisal of potential danger.

LIMITATIONS

The goal of the present study was to investigate the specific effects of REM-D on emotional reactivity. Therefore, our results do not rule out the possibility that other sleep stages may also contribute to normal emotional functioning.

Present findings should be taken as preliminary given the small number of subjects tested. However, the use of a within-subject design allowed for a larger statistical power than that which would have resulted from a cross-sectional comparison. Furthermore, regression analyses performed with all 19 subjects largely confirmed the results obtained in the group-based (categorical) analyses. In order to avoid increased between-subject variability due to sex differences in emotional processing (Stevens and Hamann, 2012), our sample was restricted to male participants. Future studies should be conducted in women to determine to what extent the present results generalize to the entire population.

Another potential limitation of the present study is the small number of slices acquired during fMRI scanning, due to technical reasons, reducing the spatial resolution of the observed activations. Nonetheless, we ensured that coverage included regions relevant for the task. Furthermore, as the same slice location was used for all subjects (see Materials and Methods) any differences (or lack thereof) observed between sessions or groups could not be attributed by differences in signal strength or coverage.

CONCLUSION

These preliminary results tentatively suggest that REM-D interferes with the brain's capacity to reestablish the adequate excitability necessary for coping with emotional events that may occur during the following day. REM-D effects on emotional reactivity deserve further research, given the chronic sleep restriction so common in modern society that prominently impacts on the amount of REM sleep, as well as for better understanding the role of REM sleep in mood disorders, such as depression and post-traumatic stress disorder, in which repetition of dream content has been interpreted as the need to reprocess an unassimilated material or to the reactivation of a high excitable circuit by REM sleep endogenous activation.

In order to confirm and generalize the findings obtained here, it would be important for future research to examine whether REM-D also affects emotional reactivity in women and to auditory stimuli and whether differences would be observed, in this case, in the auditory cortex, as was the case with visual areas in the present study. In addition, it would be interesting to assess the effects of REM-D on other aspects of emotional processing and social functioning.

In conclusion, the present findings represent, to the best of our knowledge, the first direct evidence for enhanced emotional reactivity to threatening stimuli after REM-D in humans, and for a role, specific for REM sleep, in modulating the neural substrates for emotional responsiveness.

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Regulation of fear memory by glucocorticoid and cholinergic receptors within the dorsal striatum

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A commentary on

Glucocorticoid-cholinergic interactions in the dorsal striatum in memory consolidation of inhibitory avoidance training
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A prevailing view regarding the systems neurobiology of memory formation is that different types of memory are mediated by relatively independent brain systems. In particular, the early phase of processing of declarative memories is thought to rely mostly on the dorsal hippocampus and related temporal lobe areas, while the dorsal striatum is proposed to mediate the formation of memories for procedures and habits. For example, pharmacological stimulation of the dorsal hippocampus enhances memory in water maze and radial maze tasks when training protocols based on spatial memory are used, whereas stimulation of the dorsal striatum enhances procedural versions of these tasks that use visible cues during training (Packard and White, 1991; Packard and Teather, 1998; for a recent review, see Packard and Goodman, 2012).

The consolidation of memory for inhibitory avoidance (IA), a widely used model of fear-motivated conditioning, crucially depends on neurotransmitter and protein kinase signaling, protein synthesis, and gene expression in the dorsal hippocampus (Izquierdo and Medina, 1997). In IA training, rats or mice learn, typically after a single-trial, to associate a particular location in a training box (a grid floor or a dark compartment) with an aversive stimulus (a mild footshock). Although several authors have considered IA an instrumental learning task, in which the animal learns to avoid the behavior of stepping down or stepping through to the shock compartment (Wilensky et al., 2000), the available evidence indicates that IA can be best described as a type of contextual fear conditioning (CFC), in which a novel context (an area within the training box) is associated with a conditioned stimulus (CS), i.e., a footshock. Consistent with this view, formation of memory for avoiding the footshock compartment requires contextual information, but not instrumental or procedural learning during training (Vazdarjanova and McGaugh, 1998; Roesler et al., 2001). In addition, similarly to what is observed in experiments using classic CFC (Young et al., 1994), IA training can be experimentally divided into two components: (1) context learning, which depends on *N*-methyl-D-aspartate (NMDA) glutamate receptors in the dorsal hippocampus, and (2) context-footshock association, which is impaired by NMDA receptor blockade in the basolateral amygdala (BLA) but not in the hippocampus (Roesler et al., 1998, 2003).

According to the established view of multiple memory systems, one could assume that the dorsal striatum would be important for the processing of memories for tasks based on procedural learning, but not for the initial learning of single-trial fear-motivated tasks. However, evidence dating back from the 1960s suggests that the striatum may play a more general role in memory formation in tasks including IA. Permanent lesions or reversible inactivation of the dorsal striatum, or pharmacological manipulation of specific striatal neurochemical systems including cholinergic receptors were shown to affect the formation of memory for single-trial IA learning (Kirkby and Kimble, 1968; Haycock et al., 1973; Prado-Alcalá et al., 1975, 1980). More recently, Quirarte and colleagues reported that infusion of the glucocorticoid corticosterone into the dorsal striatum enhanced consolidation of IA in rats (Medina et al., 2007). Glucocorticoids, acting on neuronal glucocorticoid receptors (GRs) in brain areas including the dorsal hippocampus and the BLA, are among the main endogenous modulatory systems involved in enhancing the consolidation of memories associated with emotionally arousing events (for reviews, see Roozendaal, 2000; de Quervain et al., 2009). Somewhat surprisingly, intra-striatal glucocorticoid administration failed to affect memory when contextual and footshock components of IA training were dissociated, suggesting that IA might involve instrumental memory aspects that are selectively regulated by striatal GRs (Medina et al., 2007). An alternative possibility would be that GRs in the striatum modulate specific aspects of IA related to the integration between context and footshock that are absent when the two components are presented separately in distinct training trials.

In a study published in *Frontiers in Behavioral Neuroscience* (volume 6, article 33), Quirarte and colleagues (Sánchez-Resendis et al., 2012) extend those findings by showing a novel functional interaction between GRs and cholinergic receptors within the dorsal striatum that influences IA consolidation. First, they found that an infusion of the muscarinic cholinergic receptor (mAchR) agonist oxotremorine into the dorsal striatum after training enhanced retention of memory for single-trial IA. They then went on to show that the oxotremorine-induced memory enhancement was blocked by either systemic or

intra-striatal administration of glucocorticoid signaling inhibitors. Finally, the memory-enhancing effect of intra-striatal corticosterone was blocked by co-infusion of the mAchR antagonist scopolamine. Together, these results indicate that two-way functional interactions between GRs and mAchRs within the dorsal striatum regulate the consolidation of memory for single-training IA.

Some aspects of the findings reported by Sánchez-Resendis et al. (2012) can be highlighted. First, they provide further evidence for an important role of the dorsal striatum in the modulation not only of typical procedural or habit memories, but also of consolidation of a memory for a single-trial, fear-motivated, hippocampus dependent type of conditioning. It would be interesting to examine whether similar effects of striatal manipulation are found when IA retention is measured after a training protocol that certainly does not include instrumental or procedural responses (i.e., when the animals, without having received context preexposure, are put directly into the footshock compartment during training, rather than stepping down or through the footshock area; Roesler et al., 2001). These experiments could help clarify whether the striatum modulates procedural and instrumental aspects of IA training.

Second, this study reveals a novel interaction between GRs and mAchRs within the striatum in modulating memory. A requirement of cholinergic receptors for GR-induced enhancement of memory consolidation had been previously shown in the BLA (Power et al., 2000). It is possible that the dorsal striatum, which has connections with both the BLA and the hippocampus, shares more memory-modulatory functions and mechanisms with these areas than previously thought. From a translational perspective, understanding how neuromodulatory systems within the striatum regulate memory formation might have implications for research on the neuropsychiatric aspects, including cognitive impairments, of neurodegenerative disorders that involve abnormalities in striatal function, such as Huntington's and Parkinson's diseases.

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Glucocorticoid-cholinergic interactions in the dorsal striatum in memory consolidation of inhibitory avoidance training

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Extensive evidence indicates that glucocorticoid hormones act in a variety of brain regions to enhance the consolidation of memory of emotionally motivated training experiences. We previously reported that corticosterone, the major glucocorticoid in the rat, administered into the dorsal striatum immediately after inhibitory avoidance training dose-dependently enhances memory consolidation of this training. There is also abundant evidence that the intrinsic cholinergic system of the dorsal striatum is importantly involved in memory consolidation of inhibitory avoidance training. However, it is presently unknown whether these two neuromodulatory systems interact within the dorsal striatum in the formation of long-term memory. To address this issue, we first investigated in male Wistar rats whether the muscarinic receptor agonist oxotremorine administered into the dorsal striatum immediately after inhibitory avoidance training enhances 48 h retention of the training. Subsequently, we examined whether an attenuation of glucocorticoid signaling by either a systemic administration of the corticosterone-synthesis inhibitor metyrapone or an intra-striatal infusion of the glucocorticoid receptor (GR) antagonist RU 38486 would block the memory enhancement induced by oxotremorine. Our findings indicate that oxotremorine dose-dependently enhanced 48 h retention latencies, but that the administration of either metyrapone or RU 38486 prevented the memory-enhancing effect of oxotremorine. In the last experiment, corticosterone was infused into the dorsal striatum together with the muscarinic receptor antagonist scopolamine immediately after inhibitory avoidance training. Scopolamine blocked the enhancing effect of corticosterone on 48 h retention performance. These findings indicate that there are mutual interactions between glucocorticoids and the striatal cholinergic system in enhancing the consolidation of memory of inhibitory avoidance training.

Keywords: glucocorticoid receptor, caudate nucleus, corticosterone, oxotremorine, scopolamine, stress hormone

INTRODUCTION

It is well established that glucocorticoid hormones (corticosterone in rodents, cortisol in humans), released from the adrenal cortex during stressful episodes, act in a neurocircuitry of interconnected brain regions to enhance the consolidation of memory of emotionally arousing training experiences (de Kloet et al., 1999; Roozendaal, 2002; McGaugh, 2004; Miranda et al., 2008; Roozendaal et al., 2009; Joëls et al., 2011; Schwabe et al., 2012). For example, corticosterone or a specific glucocorticoid receptor (GR) agonist administered into either the basolateral complex of the amygdala (BLA) or hippocampus is known to enhance memory consolidation of inhibitory avoidance training or of other training with a strong contextual component (Roozendaal and McGaugh, 1997a,b). Recently, we reported that the dorsal striatum is another target structure for glucocorticoids in modulating memory consolidation of inhibitory avoidance training (Medina et al., 2007). However, as glucocorticoid infusions into the dorsal striatum failed to enhance memory of either the footshock or contextual aspects of inhibitory avoidance training (Medina et al., 2007), these findings suggest that corticosterone might act within the dorsal striatum to specifically enhance the consolidation of memory of procedural aspects of inhibitory avoidance training, consistent with the evidence that the dorsal striatum is crucially involved in memory formation of procedural or non-declarative training (Packard and White, 1991; McDonald and White, 1993; Packard et al., 1994; Packard and Knowlton, 2002; Prado-Alcalá et al., 2003; White, 2009).

One of the neurotransmitter systems of the dorsal striatum that has received most attention is its intrinsic cholinergic system. On the one hand, it has been shown that striatal cholinergic interneurons are involved in motor control, as seen in humans where dysfunction of this complex system leads to movement disorders such as Huntington's and Parkinson's disease (Sandberg et al., 1984; Galarraga et al., 1999; Pisani et al., 2001; Wilson, 2004; Graybiel, 2008). On the other hand, muscarinic receptor antagonists administered into the dorsal striatum are known to induce

retrograde amnesia of inhibitory avoidance training (Haycock et al., 1973; Prado-Alcalá et al., 1980) in a time- and dose-dependent fashion (Prado-Alcalá et al., 1984b, 1985; Giordano and Prado-Alcalá, 1986), whereas the intra-striatal administration of muscarinic receptor agonists improves retention of this training (Solana-Figueroa and Prado-Alcalá, 1990). Additional support for the view that the striatal cholinergic system modulates memory consolidation came from experiments indicating that local activation or blockade of the cholinergic system either enhances or impairs memory of active avoidance, lever pressing, and autoshaping (Neill and Grossman, 1970; Prado-Alcalá et al., 1984a; Bermúdez-Rattoni et al., 1986).

Several findings indicate that stress exposure influences activity of the cholinergic system in the brain (Mark et al., 1996; Ortega et al., 1996; Gold, 2003) as well as that the stress associated with certain training procedures can alter cholinergic activity (Tajima et al., 1996; Orsini et al., 2001; Helm et al., 2004). However, much less is known of whether such stress effects on cholinergic activity are mediated by glucocorticoid actions. Given that both glucocorticoids and the cholinergic system play an important role within the dorsal striatum in memory consolidation, the present study investigated whether glucocorticoids and the striatal cholinergic system interact in modulating memory consolidation of inhibitory avoidance training. To address this issue, we first investigated whether the muscarinic receptor agonist oxotremorine administered into the dorsal striatum immediately after inhibitory avoidance training enhances the consolidation of memory of the training experience. Subsequently, we examined whether either systemic administration of the corticosterone-synthesis inhibitor metyrapone, suppressing the endogenous corticosterone response, or an infusion of the GR antagonist RU 38486 directly into the dorsal striatum would block the enhancing effect of oxotremorine on inhibitory avoidance memory. In the last experiment, we used a complementary approach and investigated whether a blockade of muscarinic receptors in the dorsal striatum after inhibitory avoidance training would prevent the memory enhancement induced by corticosterone.

MATERIALS AND METHODS

ANIMALS

Adult male Wistar rats (250–350 g at the time of surgery), obtained from the breeding colony of the Instituto de Neurobiología, Universidad Nacional Autónoma de México, were housed individually in acrylic cages with food and tap water available *ad libitum*. They were maintained on a 12/12 h light-dark cycle (lights on at 7:00 h) and a constant room temperature of 21°C. The rats were randomly assigned to the different experimental groups, with initial sample sizes ranging from 10 to 12 rats per group. All experimental procedures were approved by the Animal Ethics Committee of the Instituto de Neurobiología, Universidad Nacional Autónoma de México, and were in compliance with the "Principles of Laboratory Animal Care" of the National Institutes of Health.

SURGERY

Animals, adapted to the colony room for at least one week, were anesthetized with sodium pentobarbital (50 mg/kg of body weight, ip) and given atropine sulfate (0.4 mg/kg, ip) to maintain respiration. The skull was positioned in a stereotaxic frame (Stoelting, Co. IL) and two stainless-steel guide cannulae (11 mm long, 23 gauge) were implanted into the anterior division of the dorsal striatum (coordinates: anteroposterior, 0.0 mm from Bregma; mediolateral, ±3.2 mm from midline; dorsoventral, 4.2 mm below skull surface, incisor bar −3.3 mm from interaural line) according to the atlas of Paxinos and Watson (2005). The cannulae were affixed to the skull with two anchoring screws and dental cement. Stylets (11 mm long 00-insect dissection pins) were inserted into each cannula to maintain patency and were removed only for the infusion of drugs. After surgery, the rats received a subcutaneous 1 ml injection of saline to prevent dehydration and were retained within an incubator until recovered from anesthesia and were then returned to their home cages. The rats were allowed to recover for a minimum of seven days before initiation of training and were handled three times for 3 min each during this period to accustom them to the infusion procedures.

INHIBITORY AVOIDANCE APPARATUS AND PROCEDURES

The rats were trained and tested in an inhibitory avoidance apparatus consisting of a trough-shaped alley (20 cm wide at the top and 8 cm wide at the bottom) with two distinct compartments of the same size (30 × 30 × 30 cm), separated by a sliding door. The starting compartment had walls and lid made of red-colored acrylic with a floor of stainless steel bars (6 mm in diameter, separated by 9 mm) and was illuminated by a 10-W light bulb. The shock compartment was made of two electrifiable stainless steel plates and was not illuminated with its end walls and lid constructed of red-colored acrylic. In the middle of the floor, a 1.5 cm slot separated the two stainless steel plates that made up the walls and floor. The apparatus was located inside a dark, sound-attenuated room provided with background masking noise (San Diego Instruments).

For training, the rat was placed into the starting compartment of the apparatus and 10 s later the sliding door was opened and the latency to enter the dark compartment was recorded. After the animal stepped into the shock compartment with all four paws, the door was closed and a single, inescapable footshock (0.60 mA, 1 s) was delivered using a precision-regulated animal shock generator (Coulbourn Instruments, USA). Automated equipment controlled the duration of the footshock and measured the rat's latency to cross from one compartment to the other. The rat was removed from the shock compartment 10 s after termination of the footshock and, after drug treatment, returned to the home cage. On the 48 h retention test, as on the training session, the latency to re-enter the shock compartment with all four paws (maximum latency of 600 s) was recorded and used as a measure of retention. Longer latencies were interpreted as indicating better retention. Shock was not administered on the retention test trial.

DRUG AND INFUSION PROCEDURES

The muscarinic receptor agonist oxotremorine (0.15, 0.30, 0.45, 0.60, or 1.0 μg in 1 μl, Sigma-Aldrich) was dissolved in sodium phosphate buffer, pH 7.4, and infused into the dorsal striatum immediately after inhibitory avoidance training. Bilateral infusions of drug, or an equivalent volume of vehicle, were made using

30-gauge injection needles connected to 10 μl Hamilton microsyringes by polyethylene (PE-20) tubing, driven by an automated microinfusion pump (model 220i, WPI). The injection needles protruded 2 mm beyond the tip of the cannula and an injection volume of 1 μl per hemisphere was delivered over 1 min. The injection needles were retained within the cannulae for an additional minute following drug infusion to maximize diffusion and prevent backflow of drug into the cannulae.

For adrenocortical suppression, the 11-β-hydroxylase inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone; 50 mg/kg; Sigma-Aldrich) was injected subcutaneously in a volume of 2 ml/kg 90 min before training. Metyrapone was first dissolved in 100% polyethylene glycol and subsequently diluted with 0.9% saline to reach the appropriate concentration. The final concentration of polyethylene glycol was 40%. The vehicle control contained the same polyethylene glycol concentration. The dose of metyrapone was selected on the basis of previous findings indicating that it effectively blocks stress-induced increases in circulating levels of corticosterone without affecting basal levels (Roozendaal et al., 1996). In this study we did not measure corticosterone levels because there is evidence showing that the dose of metyrapone that we used (50 mg/kg) induced a significant reduction of peripheral corticosterone levels and produces memory impairment in different tasks such as passive avoidance in chicks (Loscertales et al., 1997), conditioned taste aversion (Miranda et al., 2008), spatial memory (Akirav et al., 2004), and blocks the memory enhancing effects of amphetamine or ephinephrine (Roozendaal et al., 1996) as well as the memory facilitation by stressful conditions during learning (Conboy and Sandi, 2010).

The GR antagonist RU 38486 (17β-hydroxy-11β-(4-dimethylaminophenyl)-17α-(1-propynyl)-estra-4,9-dien-3-one; 10 ng in 1 μl; Sigma-Aldrich) was microinjected into the dorsal striatum 15 min before training. RU 38486 was first dissolved in 100% ethanol and subsequently diluted in saline. The final ethanol concentration was 2%. The dose of RU 38486 was selected on the basis of previous findings indicating that, when administered into the dorsal striatum, it blocks memory enhancement of inhibitory avoidance training induced by concurrently administered corticosterone (Medina et al., 2007).

For the last experiment, corticosterone (10 or 30 ng in 1 μl; Sigma-Aldrich) was administered into the dorsal striatum together with the muscarinic receptor antagonist scopolamine (30 μg; Sigma-Aldrich) immediately after inhibitory avoidance training. Corticosterone and scopolamine were dissolved in a vehicle containing 2% ethanol in phosphate buffer, pH 7.4. The dose of corticosterone was selected on the basis of prior experiments (Medina et al., 2007).

HISTOLOGY

After completion of behavioral testing, the rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with isotonic saline followed by 4% formaldehyde. After decapitation, the brains were removed and immersed in a 4% formaldehyde solution for at least five days. Sections of 50 μm were cut on a cryostat and stained with cresyl-violet. The sections were examined under a light microscope, and the location of injection needle tips within the dorsal striatum was determined. Histological analysis revealed that the injection needle tips of seven rats were not located within the boundaries of the targeted area. The data of these animals were not included in the statistical analyses. **Figure 1** shows the location of the injection needle tips within the dorsal striatum of rats included in the statistical analyses.

STATISTICS

Data are presented as median + interquartile ranges. Inhibitory avoidance training and retention latencies were analyzed with independent Kruskal–Wallis analyses of variance. When appropriate, Mann–Whitney U-tests were used to make comparisons between any two groups. A probability level of <0.05 was accepted as statistical significance. The number of rats per group is indicated in the figure legends.

RESULTS

IMMEDIATE POST-TRAINING INFUSIONS OF OXOTREMORINE INTO THE DORSAL STRIATUM DOSE-DEPENDENTLY ENHANCE INHIBITORY AVOIDANCE MEMORY

The first experiment investigated whether bilateral infusions of the muscarinic receptor agonist oxotremorine (0.15, 0.3, 0.45, 0.6

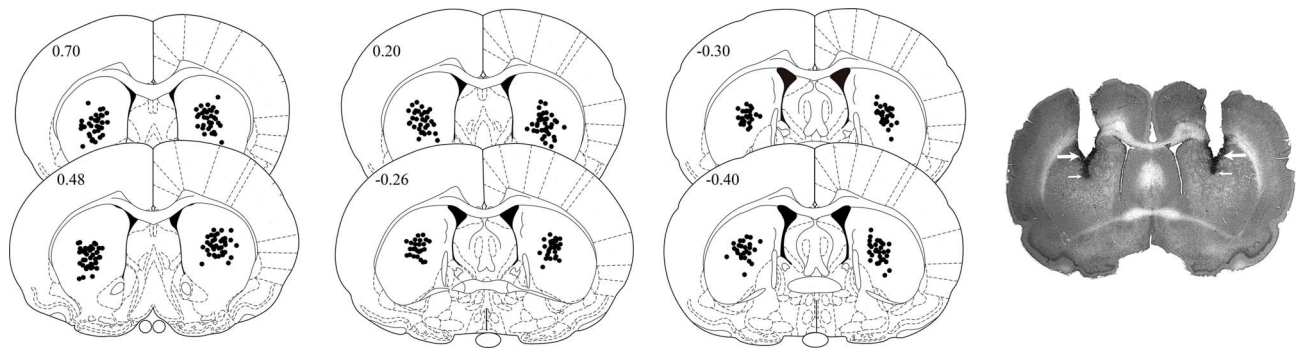

FIGURE 1 | Diagrams and photomicrograph illustrating the placement of injection needle tips within the dorsal striatum of rats trained on the inhibitory avoidance task. Large arrows point to the tracks left by the cannula tips and small arrows point to those left by the injection needle tips. Anterior-posterior coordinates are shown in mm from Bregma. Redrawn from Paxinos and Watson (2005).

or 1.0 μg in 1 μl) given immediately after inhibitory avoidance training enhance performance on a 48 h retention test. Entrance latencies during the training trial, before footshock or drug treatment, did not differ among groups [$H_{(5)} = 5.15$, $P = 0.39$; data not shown]. As is shown in **Figure 2**, Kruskal–Wallis test for 48 h retention latencies revealed a significant group effect [$H_{(5)} = 18.24$, $P < 0.005$]. Oxotremorine in a dose of 0.3 μg produced significantly longer retention latencies than those of rats treated with vehicle ($P < 0.05$). Lower or higher doses of oxotremorine did not alter retention performance.

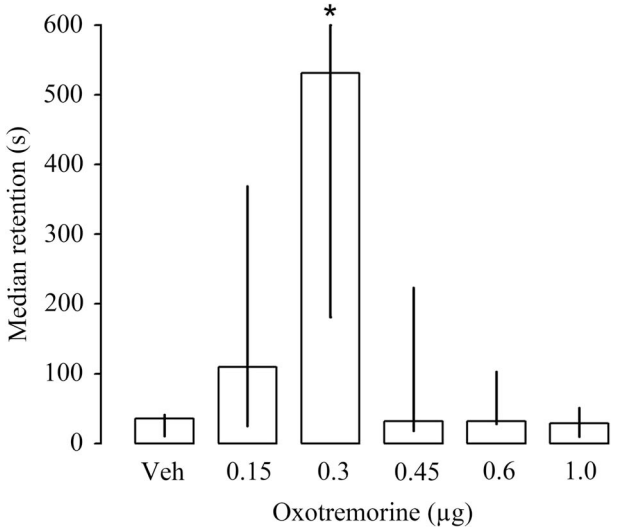

FIGURE 2 | Median latencies and interquartile ranges, in seconds on the 48 h inhibitory avoidance retention test of rats given immediate post-training infusions of either vehicle (Veh) or the muscarinic receptor agonist oxotremorine (0.15, 0.3, 0.45, 0.6, and 1.0 μg) into the dorsal striatum. The group that received the 0.3 μg dose of oxotremorine showed longer retention latencies. *$P < 0.05$ as compared to all other groups ($N = 10$ rats/group).

OXOTREMORINE-INDUCED MEMORY ENHANCEMENT DEPENDS ON CONCURRENT GLUCOCORTICOID ACTIVATION

In this experiment we investigated whether an attenuation of glucocorticoid signaling alters the memory-enhancing effects of post-training muscarinic cholinergic activation within the dorsal striatum. In the first part, rats were injected subcutaneously with the corticosterone-synthesis inhibitor metyrapone (50 mg/kg) or vehicle 90 min before inhibitory avoidance training, followed by a bilateral intra-striatal infusion of oxotremorine (0.3 or 1.0 μg) immediately after the training trial. Entrance latencies during training did not differ among groups [$H_{(5)} = 6.45$ $P = 0.26$]. However, there were significant group differences in 48 h retention latencies [$H_{(5)} = 20.48$, $P = 0.001$]. Consistent with the findings of the first experiment, Mann–Whitney U-tests indicated that rats treated with the 0.3 μg dose of oxotremorine had longer retention latencies than rats administered vehicle or the higher dose of oxotremorine (P's ranging from 0.05 to 0.005). Metyrapone treatment blocked the retention enhancement induced by intra-striatal oxotremorine administration and retention latencies of the metyrapone-oxotremorine (0.3 μg) group were significantly shorter than those of the vehicle-oxotremorine (0.3 μg) group ($P < 0.002$) (**Figure 3A**).

In the second part of the experiment, the GR antagonist RU 38486 (10 ng) was administered bilaterally into the dorsal striatum 15 min before training, followed by oxotremorine (0.3 or 1.0 μg) immediately after the training. Entrance latencies during the training trial did not differ among groups [$H_{(5)} = 7.61$ $P = 0.17$]. During 48 h retention testing, significant group differences were found [$H_{(5)} = 17.49$, $P < 0.005$]. Rats treated with

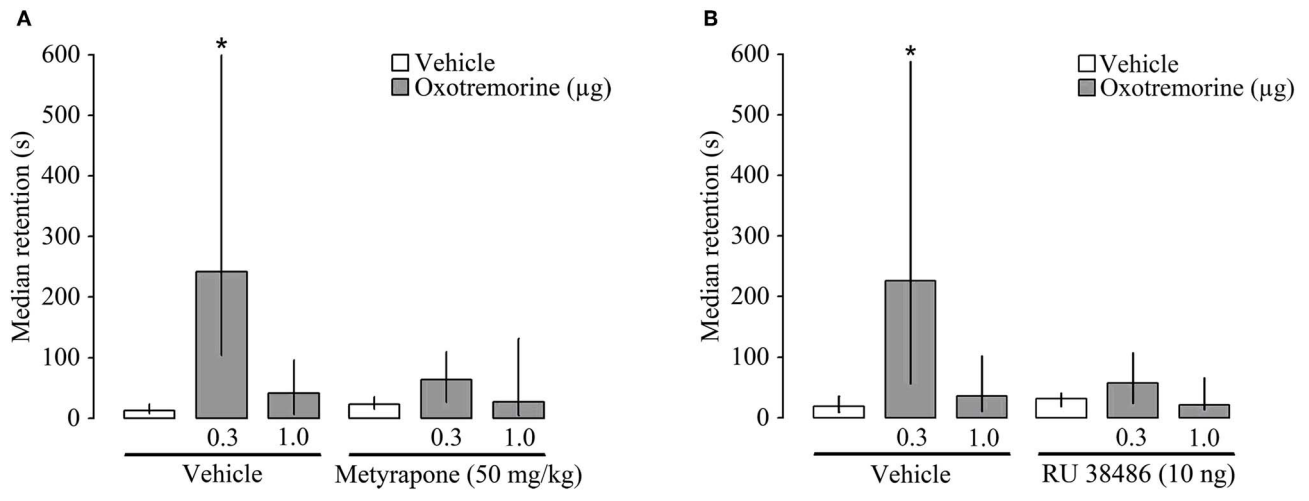

FIGURE 3 | Median latencies and interquartile ranges, in seconds on the 48 h inhibitory avoidance retention test. (A) Post-training infusion of the muscarinic receptor agonist oxotremorine (0.3 μg) into the dorsal striatum enhanced 48 h retention performance and a pre-training systemic administration of the 11β-hydroxylase inhibitor metyrapone (50 mg/kg) blocked this retention enhancement. **(B)** The glucocorticoid receptor (GR) antagonist RU 38486 (10 ng) administered into the dorsal striatum 15 min before inhibitory avoidance training also blocked the memory-enhancing effect of oxotremorine (0.3 μg) given immediately posttraining into the dorsal striatum. *$P < 0.05$ as compared to the vehicle group ($N = 10$ rats/group).

the lower dose of oxotremorine (0.3 μg) had significantly longer retention latencies than rats treated with either vehicle or the higher dose of oxotremorine (P's < 0.002 and 0.05, respectively). In rats also administered RU 38486, the memory-enhancing effect of oxotremorine was abolished and retention latencies of the RU 38486-oxotremorine (0.3 μg) group were significantly shorter than those of the vehicle-oxotremorine (0.3 μg) group ($P < 0.05$) (**Figure 3B**).

CORTICOSTERONE-INDUCED MEMORY ENHANCEMENT DEPENDS ON CHOLINERGIC ACTIVITY WITHIN THE DORSAL STRIATUM

To explore further the nature of the interaction between glucocorticoids and the cholinergic system in memory consolidation, corticosterone (10 or 30 ng) was administered bilaterally into the dorsal striatum either alone or together with the muscarinic receptor antagonist scopolamine (30 μg) immediately after inhibitory avoidance training. Entrance latencies during the training trial, before footshock or drug treatment, did not differ among groups [$H_{(5)} = 5.15$ $P = 0.40$]. As is shown in **Figure 4**, Kruskal–Wallis analysis revealed significant differences in 48 h retention latencies [$H_{(5)} = 11.28$, $P < 0.05$]. The 10 ng dose of corticosterone, but not the 30 ng dose, enhanced retention latencies ($P < 0.05$). However, scopolamine treatment blocked the memory-enhancing effect of corticosterone. Retention latencies of rats given corticosterone (10 ng) together with scopolamine were significantly shorter than those of rats given that dose of corticosterone alone ($P < 0.05$).

DISCUSSION

The present findings indicate that glucocorticoids and the cholinergic system of the dorsal striatum interact in enhancing the consolidation of memory of inhibitory avoidance training. Microinfusion of the muscarinic receptor agonist oxotremorine into the dorsal striatum immediately after inhibitory avoidance training dose-dependently enhanced 48 h retention performance. However, and importantly, an attenuation of glucocorticoid signaling with either a systemic administration of the corticosterone-synthesis inhibitor metyrapone or an intra-striatal administration of the GR antagonist RU 38486 blocked the memory-enhancing effect of oxotremorine. Further, we found that corticosterone enhanced memory when infused into the dorsal striatum after inhibitory avoidance training and that this corticosterone effect was blocked in rats concurrently administered the muscarinic receptor antagonist scopolamine.

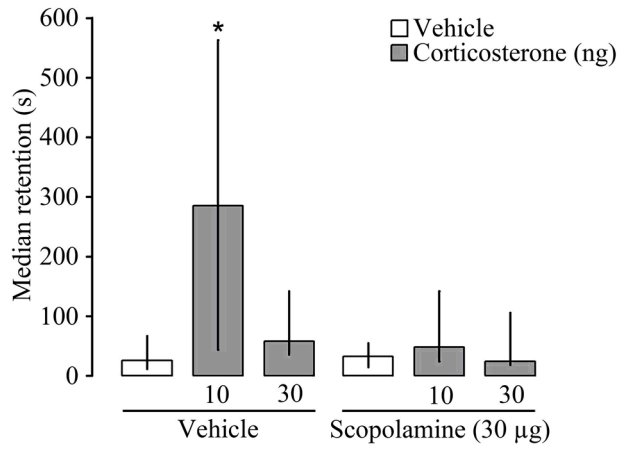

FIGURE 4 | Median latencies and interquartile ranges, in seconds on the 48 h inhibitory avoidance retention test. The muscarinic receptor antagonist scopolamine (30 μg) administered into the dorsal striatum immediately after inhibitory avoidance training blocked the memory-enhancing effect of concurrently administered corticosterone *$P < 0.05$ as compared to the vehicle group (N = 8–11 rats/group).

The current finding that oxotremorine administered into the dorsal striatum after inhibitory avoidance training enhanced 48 h retention performance is consistent with extensive evidence from other studies indicating that post-training activation of muscarinic receptors in this brain region enhances retention of inhibitory avoidance training (Solana-Figueroa and Prado-Alcalá, 1990; Ortega et al., 1996; Packard et al., 1996) or of training on several other learning tasks (Brimblecombe, 1964; Baratti et al., 1979), whereas a post-training blockade of muscarinic receptors impairs retention of inhibitory avoidance and other training (Prado-Alcalá et al., 1984b; Giordano and Prado-Alcalá, 1986). Similarly, our finding that post-training infusions of corticosterone into the dorsal striatum enhance retention of inhibitory avoidance training is consistent with other recent findings from our laboratory (Medina et al., 2007). As these memory-enhancing drugs were administered after the training trial, non-specific drug influences on the acquisition (i.e., footshock sensitivity, locomotion, attention, arousal, etc.) are excluded. Also, because these drugs are effective at enhancing long-term retention only when given within several hours after the training experience (Flood et al., 1978; Giordano and Prado-Alcalá, 1986; Sandi and Rose, 1994), the present findings strongly suggest that these drug effects on retention performance are due selectively to influences on the consolidation of long-term memory.

The aim of the present experiments was to investigate whether the glucocorticoid and cholinergic systems of the dorsal striatum interact in modulating memory consolidation of inhibitory avoidance training. The interest of this question stems from previous work indicating that stressful stimulation, either exogenously or induced by the training procedure, can alter cholinergic activity in the brain. Stressful stimuli are known to promote the release of acetylcholine in the hippocampus (Mark et al., 1996), which might directly contribute to the enhancing effects of stress on synaptic plasticity and memory (Shinoe et al., 2005). In addition to such immediate effects of stress-induced acetylcholine release on synaptic plasticity, stressful experiences might also regulate synaptic plasticity in a slower fashion by increasing, for example, the genetic expression of cholinergic nicotinic (Takita and Muramatsu, 1995) and muscarinic receptors (Takayama et al., 1987; Mizukawa et al., 1989; Gonzalez and Pazos, 1992; Kaufer et al., 1998; Brand et al., 2008). Our findings indicating that a blockade of glucocorticoid signaling prevented memory enhancement induced by oxotremorine and, conversely, that a blockade of muscarinic receptors prevented memory enhancement induced by corticosterone, suggest that glucocorticoids might be involved in mediating at least some of these stress effects on the cholinergic system.

There is some evidence in other brain regions indicating interactions between glucocorticoids and the cholinergic system. An *in vivo* microdialysis study reported that complete removal of glucocorticoids by adrenalectomy enhanced hippocampal levels of acetylcholine in response to potassium chloride stimulation (Mizoguchi et al., 2008). Another study investigated the interaction between glucocorticoids and the cholinergic system within the BLA in enhancing memory consolidation of inhibitory avoidance training (Power et al., 2000). Consistent with the current findings, blockade of muscarinic cholinergic activity within the BLA with atropine prevented the memory-enhancing effect of a concurrently administered GR agonist. In view of the evidence that glucocorticoids enhance memory consolidation by facilitating arousal-induced noradrenergic activation within the BLA (Quirarte et al., 1997; Roozendaal, 2002; Roozendaal et al., 2006), these findings were interpreted in support of the view that noradrenergic activation requires downstream cholinergic signaling within the BLA in enhancing memory consolidation (Introini-Collison and McGaugh, 1988; Decker et al., 1990). Although very little is known concerning a possible involvement of the noradrenergic system within the dorsal striatum in memory consolidation, findings from our laboratory indicate that noradrenergic activity within the dorsal striatum is essential for mediating glucocorticoid effects memory consolidation (Espinoza-González et al., 2007). Thus, such findings suggest that glucocorticoids migh interact with the cholinergic system indirectly via an activation of the noradrenergic system. However, the other findings of the current study indicating that a blockade of glucocorticoid signaling with either metyrapone or GR antagonist also prevented the memory-enhancing effect of the muscarinic receptor agonist oxotremorine cannot be readily explained by such a mechanism: previous findings indicated that a blockade of β-adrenoceptor activity within the BLA does not block the memory-enhancing effect of a muscarinic receptor agonist (Introini-Collison et al., 1996). Thus, these findings indicating that cholinergic effects on memory consolidation require concurrent glucocorticoid signaling suggest that glucocorticoids might also act within the dorsal striatum at a level that is downstream of the cholinergic system.

As glucocorticoids and muscarinic receptor agonists are known to act in many different brain regions, including the BLA, hippocampus, and prefrontal cortex, in enhancing memory consolidation of inhibitory avoidance training (Roozendaal and McGaugh, 1997b; Roozendaal et al., 1999; Barsegyan et al., 2010), what is so interesting about their effects in dorsal striatum? In inhibitory avoidance, rats learn that they receive footshock in a particular place. Previously, we developed a modified inhibitory avoidance procedure to investigate which components of the inhibitory avoidance training experience (i.e., memory of the context or the footstock were influenced by our drug manipulations). Whereas a GR agonist or oxotremorine infused into the BLA enhanced memory of both the contextual and footshock components of inhibitory avoidance training (Malin et al., 2007; Roozendaal et al., 2009), the same drugs infused into the hippocampus selectively enhanced memory of the contextual information of the task (Malin et al., 2007; Roozendaal et al., 2009). However, corticosterone administration into the dorsal striatum did not enhance memory of either the contextual or aversively motivational aspects of the task (Medina et al., 2007). In view of the evidence that the dorsal striatum is essentially involved in learning and memory of procedural or implicit forms of training (Jog et al., 1999; DeCoteau and Kesner, 2000; Packard et al., 2001; Packard and Wingard, 2004; Reiss et al., 2005; Izquierdo et al., 2006; Packard, 2009), these findings led to the suggestion that glucocorticoids might act within the dorsal striatum to selectively enhance memory consolidation of procedural aspects of inhibitory avoidance training. This view was supported by the finding that corticosterone infused into the dorsal striatum after water maze training also selectively enhanced memory of cued training, without affecting memory of spatial training (Quirarte et al., 2009). Thus, these findings indicate that although glucocorticoids and cholinergic system act in many different brain regions to enhance memory consolidation of inhibitory avoidance training, each of the brain regions might contribute in a unique fashion to enhance the consolidation of memory of specific components of information acquired during the task.

In summary, the findings presented in this work indicate that memory consolidation of inhibitory avoidance learning is facilitated by the concurrent activity of the striatal cholinergic system and glucocorticoids. Based on our prior studies, they further suggest that it is the memory of the procedural attributes of this task that was influenced by these interactions. It would seem important to determine whether the striatal cholinergic system is actually involved in the procedural aspects of inhibitory avoidance memory, as in the case of glucocorticoids in this brain region.

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Prefrontal/accumbal catecholamine system processes high motivational salience

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Motivational salience regulates the strength of goal seeking, the amount of risk taken, and the energy invested from mild to extreme. Highly motivational experiences promote highly persistent memories. Although this phenomenon is adaptive in normal conditions, experiences with extremely high levels of motivational salience can promote development of memories that can be re-experienced intrusively for long time resulting in maladaptive outcomes. Neural mechanisms mediating motivational salience attribution are, therefore, very important for individual and species survival and for well-being. However, these neural mechanisms could be implicated in attribution of abnormal motivational salience to different stimuli leading to maladaptive compulsive seeking or avoidance. We have offered the first evidence that prefrontal cortical norepinephrine (NE) transmission is a necessary condition for motivational salience attribution to highly salient stimuli, through modulation of dopamine (DA) in the nucleus accumbens (NAc), a brain area involved in all motivated behaviors. Moreover, we have shown that prefrontal-accumbal catecholamine (CA) system determines approach or avoidance responses to both reward- and aversion-related stimuli only when the salience of the unconditioned stimulus (UCS) is high enough to induce sustained CA activation, thus affirming that this system processes motivational salience attribution selectively to highly salient events.

Keywords: motivation, emotion, salience, norepinephrine, dopamine, prefrontal cortex, mesoaccumbens

INCENTIVE MOTIVATION AND MESOACCUMBENS

In the last two decades motivation theory has reached developments of paramount importance for psychology and neuroscience. Incentive motivation theory was a crucial crossroads along the way the led to such important developments. Incentive motivation concepts rose in the 1960s when several new realizations about brain and motivation led many psychologists and behavioral neuroscientists to reject simple drive and drive-reduction theories. Specific alternative theories were developed in the form of incentive motivation theories (Bolles, 1972; Bindra, 1978; Toates, 1986, 1994; Panksepp, 1998; Berridge, 2001). Three biopsychologists made major incremental contributions to its development. Bolles (1972) proposed that individuals were motivated by incentive expectancies, not by drives or drive reduction. Incentive expectancies, that Bolles called S–S* associations, were essentially learned expectations of a hedonic reward, indistinguishable from cognitive predictions. Accordingly, a predictive neutral stimulus (S), such as a light or a sound, became associated by repeated pairing with a hedonic reward that followed (S*), such as a palatable food. The S caused an expectancy of the S*. The S was, in terms of Pavlovian learning processes, a conditioned stimulus (CS or CS+), and the S* an unconditioned stimulus (UCS).

Bindra (1974, 1978) acknowledged that expectations might well be important to cognitive strategies to obtain the reward, but suggested that a CS for a reward actually evokes the same incentive motivational state normally caused by the reward itself, as a consequence of classical conditioning. The learned association does not simply cause expectation of the reward. It also causes the individual to perceive the CS as a hedonic reward, and lets the CS elicit incentive motivation just as would the original hedonic reward. That means that the CS takes on specific motivational properties that normally belong to the S* itself, and these motivational properties are specifically incentive properties. Note that this was true not only for reward S*, but also for painful S* motivation, that would be based on fear or punishment properties. Toates (1986) modified the Bolles–Bindra views suggesting that physiological depletion states could enhance the incentive value of their goal stimuli. This leads to a multiplicative interaction between physiological deficit and external stimulus, which determined the stimulus' incentive value. However, physiological deficit signals do not drive motivated behavior directly, but they are able to magnify the hedonic impact and incentive value of the actual reward (S*), and also the hedonic/incentive value of predictive stimuli for the reward (CSs). Around 1990 the incentive salience model was proposed (Berridge et al., 1989; Berridge and Valenstein, 1991) that followed Bindra–Toates rules for incentive conditioning but identifies separable brain substrates for "liking" a reward versus "wanting" the same reward. "Liking" is essentially hedonic impact—the brain reaction underlying sensory pleasure-triggered by immediate receipt of reward, for instance, a sweet taste (unconditioned "liking").

"Wanting," or incentive salience, is the motivational incentive value of the same reward (Berridge and Robinson, 1998), the incentive motivational value of a stimulus, not its hedonic impact. The important point is that "liking" and "wanting" normally go together, but they can be split apart under certain circumstances, especially by certain brain manipulations. "Liking" without "wanting" can be produced, and so can "wanting" without "liking."

Motivation can be conceptually described as a continuum along which stimuli can either reinforce or punish responses to other stimuli. Behaviorally, stimuli that reinforce are called rewarding and those that punish aversive (Skinner, 1953). Reward and aversion describe the impact a stimulus has on behavior, and provided of motivational properties, thus able to induce attribution of motivational salience.

The incentive salience model underscored the main role of dopamine (DA) function as brain mechanism of motivational processes. Indeed DA suppression leaves individuals nearly without motivation for any pleasant incentive at all: food, sex, drugs, etc., (Ikemoto and Panksepp, 1999; Naranjo et al., 2001; Berridge, 2004; Salamone et al., 2005). Thus, disruption of mesolimbic DA systems via neurochemical lesions of the DA pathway that projects to nucleus accumbens (NAc) or by receptor-blocking drugs, dramatically reduces incentive salience or "wanting" to eat a tasty reward, but does not reduce affective facial expressions of "liking" for the same reward (Pecina et al., 1997; Berridge and Robinson, 1998).

DA has a crucial role in motivational control. One type of DA neuron encodes motivational value, excited by rewarding events and inhibited by aversive or stressful events (Bromberg-Martin et al., 2010; Cabib and Puglisi-Allegra, 2012, for reviews). These neurons support brain systems for seeking goals, evaluating outcomes, and value learning. Indeed, most DA neurons are activated by reward-predicting stimuli and code bidirectional reward prediction errors (i.e., better than expected/worse than expected) in humans, monkeys, and rats (Ikemoto and Panksepp, 1999; Ikemoto, 2007; Schultz, 2007). Although discrete aversive stimuli such as air puffs, hypertonic saline, and electric shock induce activating responses in a small proportion of DA neurons in awake animals (Guarraci and Kapp, 1999; Joshua et al., 2008; Matsumoto and Hikosaka, 2009), most DA neurons are depressed by aversive stimuli (Ungless et al., 2004; Jhou et al., 2009). This response variability indicates that recorded cells are part of different, independent circuits (Margolis et al., 2006; Ikemoto, 2007; Bromberg-Martin et al., 2010). A second type of DA neuron encodes motivational salience, excited by both rewarding and aversive events (Bromberg-Martin et al., 2010).

Evidences suggest that different groups of DA neurons convey motivational signals in distinct manners (Matsumoto and Hikosaka, 2009) and the mesocorticolimbic DA system may be comprised of distinct circuits, each modified by distinct aspects of motivationally relevant stimuli, based on DA projections to NAc medial shell mediating positive stimuli, on DA projections to mpFC affected by aversive stimuli, and projections to NAc lateral shell affected by both rewarding and aversive stimuli, presumably reflecting saliency (Lammel et al., 2011). It has been shown how the VTA DA neurons may employ the convergent encoding strategy for processing both positive and negative experiences, intimately integrating with cues and environmental context (Wang and Tsien, 2011).

The mesolimbic dopaminergic system, that projects from the ventral tegmental area (VTA) neuronal cell bodies rostrally to the NAc, is a primary link in the reward pathway (Wise, 1996, 2004). However, DA release is not necessary for all forms of reward learning and may not always be "liked" in the sense of causing pleasure, but it is critical for causing goals to become "wanted" in the sense of motivating actions to achieve them (Robinson and Berridge, 1993, 2003; Berridge and Robinson, 1998; Palmiter, 2008).

A line of evidence supporting a role for DA in the motivational properties of stimuli comes from the place-conditioning paradigm (Mucha and Iversen, 1984; van der Kooy, 1987; Carr et al., 1989). This paradigm treats the increase in amount of time spent in an environment that has been paired with an UCS (either drugs or natural reinforces) as an index of the stimulus's reward properties. By contrast, if animals are repeatedly exposed to an environment paired with an aversive stimulus they will avoid the environment. In the first case we speak of conditioned place preference (CPP), in the second of conditioned place aversion (CPA). DA antagonists administered before each conditioning session with amphetamine block amphetamine-conditioned place preferences (Nader et al., 1997 for review). These results are not interpretable in terms of a general learning deficit because animals have been shown to be able to form normal CS-US associations in place conditioning with other US (Shippenberg and Herz, 1988). These findings suggest that normal DA transmission is necessary for the rewarding properties of stimuli to occur.

If the dopaminergic pathway from the VTA to the NAc is a primary link in the pathways mediating the motivational properties of stimuli (Tsai et al., 2009; Adamantidis et al., 2011), then examples of DA-independent reward should be nonexistent. There are a number of examples, however, of stimuli that possess reinforcing properties independent of DA. Thus, behavioral pharmacological experiments indicate that although increasing mesolimbic DA transmission plays an important role in the reinforcing effects of abused substances, there are also DA-independent processes that contribute significantly to the reinforcing effects of these compounds (Joseph et al., 2003; Pierce and Kumaresan, 2006 for review). For instance, DA antagonist pretreatment or 6-OHDA lesions of the NAc have been reported to have no effect on morphine or heroin self-administration (Ettenberg et al., 1982; Pettit et al., 1984; Dworkin et al., 1988), and on ethanol oral self-administration (Rassnick et al., 1993). A lack of dopaminergic involvement in cocaine place preferences (Spyraki et al., 1982; Mackey and van der Kooy, 1985) has been reported following systemic or intra-accumbens administration (Koob and Bloom, 1988; Hemby et al., 1992; Caine and Koob, 1993). Under some conditions, DA-independent opiate place preferences has been demonstrated (Mackey and van der Kooy, 1985; Bechara et al., 1992; Nader et al., 1994). Moreover, DA-deficient mice display a robust conditioned place preference for morphine in specific experimental conditions (Hnasko et al., 2005), and DA is not involved in opiate naive state (Laviolette et al., 2004; Vargas-Perez et al., 2009). A DA-independent reward mechanism for caffeine has been shown (Sturgess et al., 2010).

Dopamine D2 receptor knockout mutation in C57BL/6 mice failed to block ethanol-conditioned place preferences in ethanol-dependent and withdrawn mice (Ting-A-Kee et al., 2009). In a more "naturalistic" conditions operant place conditioning by male chemo-signal in female mice was not affected by D1 or D2 receptor antagonists (Agustin-Pavon et al., 2007). It is worth noting that VTA-mediated but DA-independent positive reinforcement has been demonstrated (Fields et al., 2007).

These examples of DA-independent motivated behavior seriously question the original DA hypothesis that suggested DA is a final common pathway in the processes mediating reinforcement.

PREFRONTAL-ACCUMBAL CATECHOLAMINE SYSTEM

About a couple of decade ago, research has pointed to prefrontal catecholamine (CA) regulation of mesoaccumbens DA transmission in response to pleasant or aversive stimuli (Le Moal and Simon, 1991). In particular, DA transmission in subcortical structures, such as the NAc, seems to be modulated by the DA mesocortical system in an inhibitory way (Ventura et al., 2004, for review), thus strongly suggesting that the mesoaccumbens DA response is inversely related to the mesocortical DA response.

Mesoaccumbens DA transmission has been suggested to be regulated by prefrontal transmission via glutamatergic projections (Carr and Sesack, 2000, for review), through activation of excitatory prefrontal-cortical projection to the VTA (Sesack and Pickel, 1990), and/or through activation of a corticoaccumbens glutamatergic projection (Taber and Fibiger, 1995). Thus, besides possible direct cortico-accumbal circuit, a cortico-(VTA)- accumbal DA network involving different brain areas, such as amygdala (Jackson and Moghaddam, 2001; Mahler and Berridge, 2011), has been proposed to have an important role on accumbal DA modulation.

In late nineties, a French study (Darracq et al., 1998) showed that prefrontal cortical norepinephrine (NE) had a pivotal role in the increased accumbal DA release induced by systemic amphetamine administration. Till that moment, brain noradrenergic system involvement in behavior control was mostly focused on Locus Coeruleus (LC) functions (Aston-Jones et al., 1999) or on emotional memory regulation by the amygdala (McGaugh, 2006). The pioneer work of Darracq and coworkers, suggested implicitly that DA transmission in the NAc could be controlled by and directly related to NE in the medial prefrontal cortex (mpFC). This view, together with the established inhibitory role of prefrontal DA on dopaminergic activity in the accumbens, suggested a possible opposite action of the two amines in the prefrontal cortex on subcortical DA transmission.

Experimental evidence from our lab on mice of C57BL/6 (C57) and DBA/2 (DBA) inbred strains supported this hypothesis. Comparative studies of neurotransmitter activity and behavior in different genetic backgrounds make available a major strategy for investigating the neural basis of drug effects related to individual differences. Mice of the DBA background have been shown to be poorly responsive to the enhancing extracellular DA induced by the psychostimulant in the NAc (shell) as well as to the stimulating/reinforcing effects of amphetamine, that are dependent on increased accumbal DA release. The opposite occurs in mice of the C57 background, which have been shown to be highly responsive to stimulating/reinforcing effects of amphetamine, as shown by increased locomotor activity or to amphetamine-induced CPP (Zocchi et al., 1998; Cabib et al., 2000). In C57, Amphetanine produces low mpFC DA and high DA in the NAc, the opposite occurs in DBA mice that show lower locomotor activity than C57and no CPP or even CPA. Moreover, selective DA depletion in the mpFC of DBA mice makes this strain similar to highly responsive C57 mice leading to high DA outflow in the NAc and hyper locomotion. Nevertheless, no differences in structure or expression of DA transporter in the NAc between C57 and DBA strains have been reported (Womer et al., 1994). These results showed that the different effects of amphetamine on accumbal DA outflow in the two backgrounds do not depend on differences in DAT related mechanisms. However, microdialysis experiments showed that amphetamine increased NE and DA outflow in the mpFC of C57 and DBA mice in a different way. While C57 showed higher NE increase than DA, DBA mice present an opposite pattern, thus indicating that NE/DA ratio induced by amphetamine is higher in C57 versus DBA. Since DA is inhibitory on DA NAc, while NE was suggested to be enabling (Darracq et al., 1998), we hypothesized that imbalanced NE/DA in the mpFC controlled DA in the NAc and related behavioral outcomes, making the C57 strain more responsive than DBA. Such a hypothesis was confirmed by subsequent experiments showing that selective prefrontal cortical NE depletion abolished the effects of amphetamine on DA in accumbens and CPP in C57 mice (Ventura et al., 2003), while selective prefrontal DA depletion (sparing NE) led to DA outflow in the NAc and behavioral outcomes in DBA mice entirely similar to those of C57 (Ventura et al., 2004, 2005).

These data suggested strongly that DA in the NAc is controlled by prefrontal cortical NE that enables it, and by DA that inhibits it. Moreover, our data indicated that prefrontal NE transmission is critical for attribution of motivational salience, as demonstrated by the impairment of amphetamine-induced CPP in mpFC NE depleted C57 mice (Ventura et al., 2003).

However, evidence in the literature (Ventura et al., 2002 for review) and results on stress obtained in our lab on C57 and DBA mice had shown that this was true also for aversive experiences (restraint, Forced Swimming), at least as far prefrontal DA control over DA in the NAc was concerned. Indeed, we found that restraint stress produced inhibition of mesoaccumbens DA release accompanied by a very fast and strong activation of mesocortical DA metabolism in C57 mice, and the opposite in mice of the DBA strain, showing a genetic control over the balance between mesocortical and mesoaccumbens DA responses to stress (Ventura et al., 2001). Moreover, C57 mice but not mice of the DBA strain showed an extremely high level of immobility on their first experience with the forced swimming test (FST) as well as immediate and strong activation of mesocortical DA metabolism and inhibition of mesoaccumbens DA metabolism and release. In addition, the behavioral and the mesoaccumbens DA responses to FST in C57 mice were reduced and reversed, respectively, by selective dopamine DA depletion in the mpFC (Ventura et al., 2002).

Prefrontal NE transmission was known to play a critical role in regulating many cortical functions, including arousal, attention, motivation, learning, memory, and behavioral flexibility (Sara and Segal, 1991; Tassin, 1998; Feenstra et al., 1999; Arnsten, 2000; Robbins, 2000; Bouret and Sara, 2004; Dalley et al., 2004; Mingote et al., 2004; Tronel et al., 2004; Aston-Jones and Cohen, 2005; Rossetti and Carboni, 2005; Lapiz and Morilak, 2006; van der Meulen et al., 2007; Robbins and Arnsten, 2009). Moreover, both rewarding/reinforcing and aversive stimuli have been shown to increase NE release in pFC (Finlay et al., 1995; Dalley et al., 1996; Goldstein et al., 1996; Jedema et al., 1999; Kawahara et al., 1999; McQuade et al., 1999; Feenstra et al., 2000; Page and Lucki, 2002; Morilak et al., 2005; Feenstra, 2007). These evidences suggested that CA prefrontal transmission could control DA in the accumbens also in stressful conditions, a hypothesis that deserved to be assessed. This was done by two independent laboratories and published in 2007. These studies showed that novel stressful experiences enhance DA release in the NAc through activation of prefrontal cortical alpha-1 adrenergic receptors (ARs) by high levels of released NE (Nicniocaill and Gratton, 2007; Pascucci et al., 2007). Indeed, the experience with a novel stressor promotes a rapid, massive, and transient increase in NE release within the mpFC which parallels the enhancement of mesoaccumbens DA release (Pascucci et al., 2007). A selective depletion of prefrontal cortical NE prevents both the cortical NE response and the increase in accumbal DA, leaving stress-induced enhancement of prefrontal cortical DA release as well as basal CAs levels unaffected (Pascucci et al., 2007). Moreover, applications of the alpha-1 AR selective antagonist benoxathian into the mpFC inhibits stress-induced DA release in the NAc dose-dependently (Nicniocaill and Gratton, 2007). Pascucci et al. (2007) also confirmed that stress-induced enhanced NAc DA release is constrained by activation of mpFC DA. Indeed, either DA depletion (Deutch et al., 1990; Doherty and Gratton, 1996; King et al., 1997; Pascucci et al., 2007) or blockade of D1 receptors by infusion of a selective antagonist in the mpFC (Doherty and Gratton, 1996) enhances stress-induced DA release in the NAc. It is known that DA in the mpFC exerts an inhibitory influence on DA release in the NAc and depletion of mesocortical DA facilitates stress-induced activation of mesoaccumbens DA release (Deutch et al., 1990; Doherty and Gratton, 1996; King et al., 1997). However, our results demonstrated that during novel stressful experiences the mpFC determines mesoaccumbens DA response through the opposing influences of NE and DA. Our data could explain why stress may be involved in different pathological conditions. Indeed, the balanced action of the two CAs in the mpFC may be required for healthy coping, whereas unbalanced action may promote hyper- or hypo-responding by mesoaccumbens DA, leading to different and even opposite behavioral disturbances.

The opposite influence exerted by mpFC NE and DA on DA transmission in the NAc during stressful experiences points to possible opposite modulation of frontal cortical glutamate (GLU) by the two CAs. Since blockade of mpFC alpha-1 ARs or D1 receptors has opposite effects on stress-induced GLU increase (Lupinsky et al., 2010), it is likely that frontal cortical NE and DA exert opposite effects on mpFC output, possibly through glutamatergic stimulation of GABA interneurons in the mpFC (Del Arco and Mora, 1999; Homayoun and Moghaddam, 2007).

The involvement of alpha1-ARs in the prefrontal NE control of DA release in the NAc during stress is consistent with evidence that a sustained increase of prefrontal cortical NE (as the one induced by stress) is capable to activate these low-affinity receptors subtypes, while mild increase is able to activate high affinity alpha2- or beta1- ARs (Ramos and Arnsten, 2007). However, the main role of alpha1-ARs in the mesoaccumbens DA activation by stress or by amphetamine (Darracq et al., 1998; Ventura et al., 2003; Nicniocaill and Gratton, 2007), and the crucial role of prefrontal NE in attribution of motivational salience to stimuli related to amphetamine, as shown by CPP study in the mouse (Ventura et al., 2003), point to a main role of these receptors in motivated behavior and coping. mpFC and NAc receive DA afferents from different populations of VTA DA cells and these are controlled by different circuits (Joel and Weiner, 1997; Carr and Sesack, 2000; Lewis and O'Donnell, 2000; Margolis et al., 2006; Lammel et al., 2008; Tierney et al., 2008). VTA also receives afferents from the central nucleus of amygdala (CeA); the inhibition of CeA, and hence of its inhibitory input to VTA, leads to an increase of NAc DA (Ahn and Phillips, 2003; Phillips et al., 2003a), suggesting that this input is part of a double inhibition mechanism (Fudge and Haber, 2000; Ahn and Phillips, 2002; Floresco et al., 2003; Fudge and Emiliano, 2003). NE afferents in the mpFC originate from the relatively small group of cells of LC (Aston-Jones et al., 1999; Valentino and van Bockstaele, 2001; Berridge and Waterhouse, 2003). LC receives strong convergent projections from the orbito-frontal and cingulate cortex, which have been suggested to drive transitions between phasic and tonic modes in NE neurons to fit the behavioral/cognitive states with environmental conditions (Aston-Jones and Cohen, 2005). LC activity is also modulated by CeA (Curtis et al., 2002) through innervation of the peri-coerulear region (Berridge and Waterhouse, 2003) and through the excitatory corticotropin-releasing hormone (Van Bockstaele et al., 2001; Bouret et al., 2003; Jedema and Grace, 2004). NE has different effects on target cortical areas depending on its concentration and on the distribution of alpha1 and alpha2 receptors (Briand et al., 2007; Arnsten, 2009). Indeed, different levels of tonic neuromodulator release affects receptors that are differentially located among cortical layers, so that a neuromodulator may differently affect its target subregions depending on the receptors it activates.

The evidences considered till now indicate that a prefrontal CA system controls DA release in the NAc, a sub-cortical area known to be involved in all motivated behavior, independently of the valence of the stimuli or experiences. Thus, a similar prefrontal-accumbal regulation has been shown for rewarding (amphetamine) or aversive (stress) stimuli. Further studies provided substantial support to this view, through experimental evidences that prefrontal cortical NE is crucial in the effects of other addictive drugs, of palatable food, and of aversive pharmacological or physical stimuli. Moreover, they demonstrated that prefrontal NE through its action on NAc DA is essential in attribution of motivational salience in specific conditions, as it will be shown in the next paragraph.

PREFRONTAL NE- ACCUMBAL DA IN MOTIVATIONAL SALIENCE ATTRIBUTION TO BOTH APPETITIVE- AND AVERSION-RELATED STIMULI

Other addictive drugs, in addition to amphetamine, increase DA release in the NAc through prefrontal NE, as shown by experiments based on intracerebral microdialysis in the mouse and on selective NE depletion in the mpFC. Selective NE depletion was carried-out by the neurotoxin 6-hydroxydopamine and pretreatment with the selective DA transporter blocker GBR-12909 that produced about 90% NE afferents destruction, with no significant effects on DA. To avoid substantial changes in receptor regulation, neurochemical and behavioral testing were carried out within one week from surgery. Morphine (Ventura et al., 2005), Cocaine (Ventura et al., 2007), ethanol (Ventura et al., 2006, in preparation) have been shown to induce dose-dependent increase of NE in the mpFC and a parallel increase of DA in the NAc. Selective prefrontal NE depletion abolished the outflow increase of both prefrontal NE and DA in the NAc, thus confirming the crucial role of NE in mpFC in accumbal DA activation induced by different classes of drugs of abuse. It is worth noting that all the assessed drugs increased DA outflow in the mpFC, that was not affected by NE depletion. However, one may hypothesize that, based on the known inhibitory role of prefrontal DA on DA release in the NAc observed in animals receiving drugs (e.g., amphetamine) or stress, the failure of DA increase in the NAc of NE mpFC depleted subjects receiving drugs was due to the prevalent inhibitory action of prefrontal DA in the absence of NE. Such a view, would affirm the crucial "promoting" role of prefrontal NE on accumbal DA, pointing, however, to a complementary role of DA in mpFC that would exert an inhibitory role leading to "flatting" accumbal DA when cortical NE is depleted. This possibility was ruled-out by complementary experiments showing that concomitant depletion of NE and DA in mpFC does not change the impaired accumbal DA release in mice receiving AMPH in comparison with animals subjected to selective NE depletion. A body of evidence suggests that DA in the prefrontal cortex is co-released with NE from noradrenergic terminals (Devoto et al., 2001, 2002). Moreover, it has been reported that DA in this brain area is normally cleared by NE transporter (Tanda et al., 1997; Moron et al., 2002). Different set of data obtained in both mice and rats, showed a lack of effects of the NE depletion on basal extracellular DA, suggesting that the likely reduction of DA released from destructed noradrenergic terminals is compensated by augmented availability of DA due to its reduced uptake from these terminals (Ventura et al., 2005; Pascucci et al., 2007). However, NE-depleted mice showed an increase of morphine-induced DA release similar to that exhibited by Sham animals, thus suggesting that prefrontal noradrenergic and dopaminergic projections are functionally uncoupled. In agreement with this observation, selective prefrontal NE depletion in rats did not affect stress-induced DA release and selective DA depletion did not affect stress-induced NE release. Taken together, these data indicate that, in both reinforcing (morphine injection) and aversive (stressful situation) conditions, NE and DA release in the mpFC are independent.

This evidence suggests that NE is a common regulating element responding to different class of stimuli to induce DA activation in the NAc, irrespective of the specific pharmacological or physiological properties of stimuli. Possible network elements have been mentioned before and will be further considered. Here, it is worth to point out that different class of pleasant stimuli as well as aversive experiences such stress are likely to activate a common prefrontal cortical-subcortical network.

The role of mesoccumbens DA system in motivation is well-established. However, whether also a system, involving prefrontal NE and accumbal DA, has a role, needs experimental support. To study incentive learning and incentive motivation, place conditioning is commonly exploited in rats and mice, but in the last species is prevalent since operant procedures that are mostly used for studying drug self-administration in rats, present a number of difficulties in mice. Anyway, this method allows attribution of motivational salience to stimuli related to either pleasant (appetitive) or aversive stimuli (US). In the first case pairings between stimuli and environment (CS) lead to place preference (CPP), while in the second produce place aversion (CPA). The process of attribution of motivational salience is measured by the preference (or the aversion) shown when a subject has to choose between the environment previously paired with the US and a neutral environment (Tzschentke, 1998; Mueller and Stewart, 2000). This method is also useful to assess relapse to previously preference (or aversion) after extinction, and is a choice method in modeling addiction (Lu et al., 2003; Shaham et al., 2003). Indeed, a before mentioned study had shown that selective prefrontal cortical NE depletion besides impairing amphetamine-induced DA outflow increase in the NAc, impaired CPP induced by the stimulant. These effects were not due to motor deficits, or learning impairments, since depleted animals were not different from sham controls in motor behavior, and, most importantly, were able of associative learning as shown by avoidance test (Ventura et al., 2003).

Moreover, these results indicate that intact prefrontal cortical NE is necessary for CPP induced by morphine, cocaine, or ethanol as well as for reinstatement (relapse) of extinguished morphine-induced CPP, and for ethanol intake in a choice test. Thus, they demonstrate that prefrontal NE is crucial for DA release in the NAc induced by addictive drugs and for attribution of motivation salience to drug-related stimuli.

However, the results concerning aversive experiences demonstrate that the noradrenergic control of accumbal DA activation is evident also for stress, suggesting a common network involved in processing both pleasant (rewarding) and aversive stimuli. To assess this hypothesis we planned two experiments. In the first one we observed that a pharmacological aversive stimulus such lithium chloride administered systemically in mice induced a clear-cut increase of NE in the mpFC and DA in the accumbens that was abolished by selective prefrontal NE depletion. Moreover, lithium induced a CPA that was abolished by prefrontal NE depletion, thus confirming that prefrontal NE is crucial for attribution of motivational salience to stimuli related to aversive experience (Ventura et al., 2007).

Next step was suggested by preliminary results obtained when we decided to assess the role of prefrontal-accumbal CA system in attribution of motivational salience to natural non-pharmacological stimuli. Previous data in the literature allowed

to hypothesize that appetitive or aversive stimuli produce a graded activation of prefrontal noradrenergic transmission, thus more salient is a stimulus stronger the prefrontal NE release will be (Feenstra et al., 2000; Ventura et al., 2008 for review). If this were the case, then prefrontal NE release could be considered an index of stimuli salience. To further support that prefrontal NE-accumbal DA system is crucial for attribution of motivational salience also for aversive stimuli we used as aversive non-pharmacological experience a stressor (intermittent lights) that could be graded in order to provide parallel effects to those of pleasant (rewarding) stimuli like palatable food before described. In place conditioning preliminary tests, in which the two stressors were compared we observed that they differ in the conditioned aversive effects, the pulsating intermittent lights being more aversive than intermittent non-pulsating lights. This result paralleled the effects of the two aversive conditions on prefrontal cortical NE release. Both lighting conditions increased prefrontal NE release, but pulsating lighting produced more pronounced increase than non-pulsating lighting. Moreover, noradrenergic response in mpFC was paralleled by graded increase of DA in the NAc (Ventura et al., in preparation).

Then, we assessed if appetitive non-pharmacological stimuli, used as US in place conditioning, required intact prefrontal NE-accumbal DA functioning for attribution of motivational salience. We have observed that mice preferred white chocolate (WCh) to Milk (MCh)-chocolate in a free choice test, a preference that was confirmed in a CPP paradigm where mice chose the environment paired with WCh in comparison with that paired to MCh-chocolate. Consistently, intracerebral microdialysis showed that exposure to WCh intake produces a higher NE release in mpFC than MCh (Ventura et al., 2008, in preparation) accompanied by a more sustained DA outflow in the NAc. These results demonstrate that prefrontal NE and accumbal DA respond to different salient stimuli, either pleasant or aversive, in a graded manner.

Incentive motivation theory has pointed to the major role of the motivational status of the organism (hungry, thirsty, tired, alert etc.) when it is confronted with a stimulus or experience. Stress has received much attention in motivation related studies, particularly those concerning addiction models, for neuroadaptation it can produce in the brain systems involved in the response to drug priming, incentive learning processes, and relapse. We wondered if pre-exposure to stressful experience could affect the "perceived" salience of the stimulus and the response of prefrontal-accumbal CA system, and if such changes could affect attribution of motivational salience in our experimental conditions. We used a food-restriction regimen as chronic stress that was also shown to change the behavioral response to amphetamine and, affect attribution of motivational salience in mice (Cabib et al., 2000; Guarnieri et al., 2011). Food-restriction (FR) led to higher NE release in mpFC and higher DA release in the NAc in comparison with control mice. This increase was similar to that shown by free-fed (Non-FR) mice exposed to WCh, thus showing that the state of the organism, as expected, influenced the response to appetitive stimuli. This effect may be ascribed obviously to the food deprivation that would make it more palatable. However, our data indicate that FR regimen is an environmental condition that affects the perceived salience, independently of food-related mechanism. Indeed, we observed that FR made the effects induced by the less salient stressor (intermittent light) similar to the effects produced in non-FR mice by the more salient stressor (pulsating intermittent light). This means that FR is capable to increase the salience of both pleasant (rewarding; food) and aversive (stressful lighting) stimuli, regardless of hunger-related mechanisms. Note that in additional experiments Sham and NE depleted mice subjected to a different no food-related chronic stressful experience (social isolation) showed similar effects to those of FR animals, thus indicating that the effect of prefrontal NE depletion on MCh-induced CPP cannot be ascribed to the homeostatic response to dietary restriction (Ventura et al., 2008). Food-restriction can also be considered as leading to a generalized drive effect (Niv et al., 2006; Phillips et al., 2007) that would "energize" motivation. This mechanism seems to depend on deprivation states. Our results, however, indicate that a generalized drive effect produced by food-restriction regimen before exposure to specific stimuli affects not only appetitive alimentary stimuli but also aversive stimuli. In fact, aversive effects of intermittent light are stronger in food-restricted than in free-fed mice. Thus, a generalized drive effect should involve common neural mechanisms regulating both appetitive and aversive experiences.

Taken together, these results show that the prefrontal-accumbal CA response is an index of the emotional/motivational impact of differently salient stimuli depending on the stimuli characteristics or on the state of the organism. The graded response of prefrontal NE was in agreement with previous results and suggested to us to ascertain the role of prefrontal-accumbal CA system in attribution of motivational salience related to differently salient stimuli. Using the experimental paradigms of other studies on the same topics, we assessed the effects of selective prefrontal NE depletion on the CA response and on attribution of motivational salience measured by place conditioning. Surprisingly, we observed that NE depletion abolished the increase of prefrontal cortical NE release and of accumbal DA, consistently with previous experiments. However, it prevented place preference (CPP) in animals exposed to WCh and in food-restricted (FR) animals exposed to milk chocolate (MCh; both conditions of high salience) but not in non-FR (Free-fed) animals exposed to MCh (low salience). Moreover, it prevented place aversion (CPA) in animals exposed to intermittent pulsating light (IPL) and in the FR animals exposed to intermittent light (IL; high salience) but not in non FR animals exposed to IL (low salience; **Figure 1).**

These results show that that pFC NE depletion affects attribution of motivational salience only when the salience of the UCS is high enough to induce sustained CA activation, thus indicating that prefrontal-accumbal CA system is involved in processing motivational salience attribution selectively when intense motivational salience is processed. Salience refers to the capability of stimuli to be arousing (Horvitz, 2000). Salient stimuli cause the reallocation of available cognitive resources in order to produce an attentional or a behavioral switch (Zink et al., 2006). The more salient the stimulus, the more likely it will lead to an

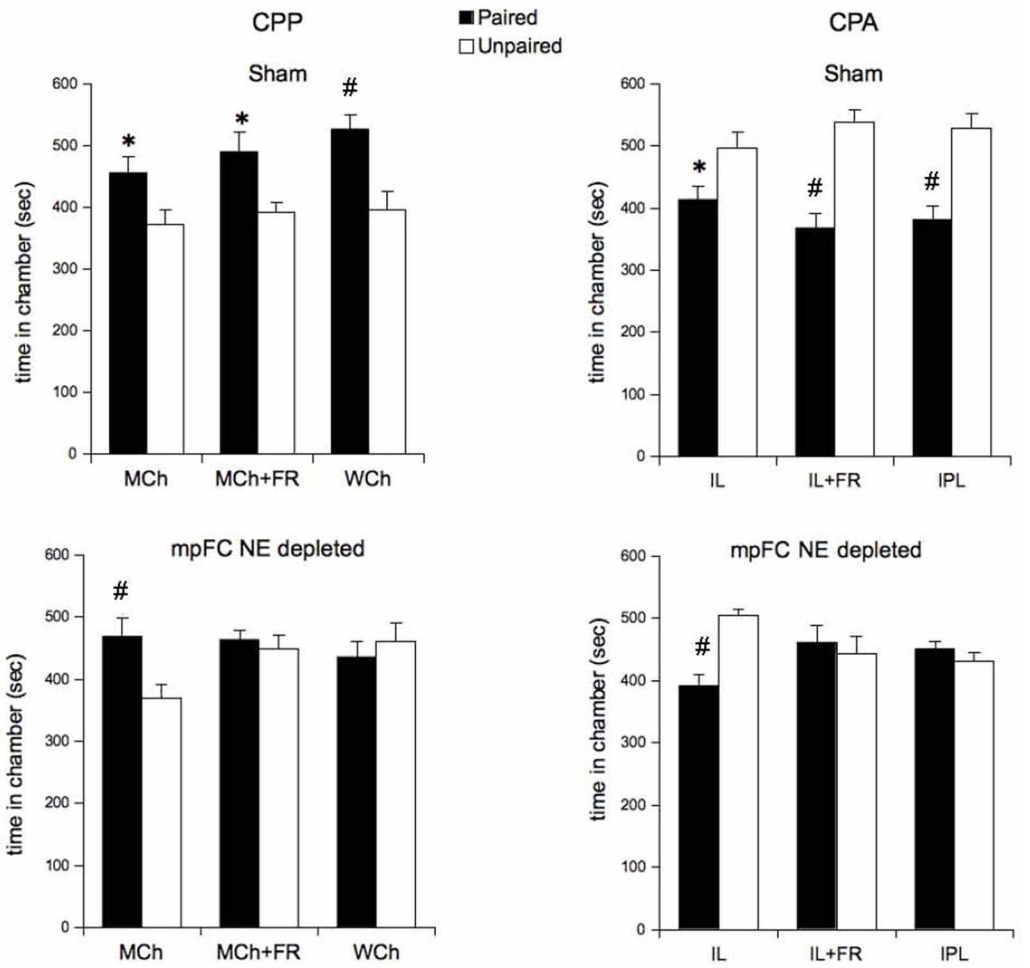

FIGURE 1 | Effects of prefrontal cortical norepinephrine depletion on conditioned place preference (CPP) induced by chocolate (milk chocolate in control, MCh; milk chocolate in food restricted MCh+FR; white chocolate in control, WCh) and conditioned place aversion (CPA) induced by light (intermittent light in control, IL; intermittent light in food restricted, IL+FR; intermittent-pulsating light in control; IPL) [Sham, norepinephrine-depleted in medial prefrontal cortex (mpFC NE Depleted)]. All data are expressed as mean (sec ± SE) time spent in Paired, and Unpaired chambers. $^{*}P < 0.05$ in comparison with the Unpaired chamber; $^{\#}P < 0.005$ in comparison with the Unpaired chamber (from Ventura et al., 2008, modified).

attentional or a behavioral switch. Recent reports in humans have shown that striatum has a major role in prompting the reallocation of resources to salient stimuli (Zink et al., 2003, 2006). However prefrontal cortex, due to its "supervisory" functions, has an unquestionable central role in attentional and motivational processing of salient stimuli.

Moreover, data indicate that ventral striatum (or NAc) and prefrontal cortex constitute a common substrate for processing both rewarding and aversive stimuli (Berridge and Robinson, 1998; Darracq et al., 1998; Becerra et al., 2001; Jensen et al., 2003; Kensinger and Schacter, 2006; Borsook et al., 2007), and neuroimaging studies in humans suggest that different areas of prefrontal cortex (O'Doherty et al., 2001; Small et al., 2001; Killgore et al., 2003; Wang et al., 2004) and of striatum (Jensen et al., 2003; Zink et al., 2006; Borsook et al., 2007) are activated by natural positive or negative salient stimuli. Again, we have previously demonstrated that intact NE prefrontal transmission is necessary for motivational salience attribution to both natural (in food restricted animals) and pharmacological reward related stimuli as well as to pharmacological aversion related stimuli through modulation of DA in NAc (Ventura et al., 2007). Therefore, it is likely that the effects of prefrontal NE depletion on CPP and CPA in animals exposed to highly salient stimuli depend on the impaired response of prefrontal-accumbal CA system, whose activation by unconditioned rewarding and aversive highly salient stimuli is a substrate for motivational salience. However, other brain areas and neurotransmitters are likely to be engaged. Thus, since amygdala is involved in pavlovian conditioning of emotional responses and plays a specific role in modulating memory for arousing experiences (Balleine, 2005; Balleine and Killcross, 2006; McGaugh, 2006), and given the complex anatomical and functional connections between this brain area and prefrontal cortex (Cardinal et al., 2002; Holland and Gallagher, 2004; Roozendaal et al., 2004) a role of a prefrontal cortex-amygdala system in the effects of

highly salient stimuli reported here must be considered (Belova et al., 2007).

CONCLUSIONS

Attribution of motivational salience is related to the salience of an UCS (Dallman et al., 2003; Pecina et al., 2006). Thus, the more salient an UCS the more likely a neutral (to-be-conditioned) stimulus will be associated with it through motivational salience attribution. Prior experience is a major determinant of the motivational impact of any given stimulus (Borsook et al., 2007) and emotional arousal induced by motivational stimuli increases the attention given to stimuli influencing both the initial perceptual encoding and the consolidation process (Anderson et al., 2006; McGaugh, 2006). We provided evidence that prefrontal-accumbal CA transmission is necessary for motivational salience attribution to both reward- and aversion-related stimuli only under those conditions able to induce stronger increases of CA outflow in response to highly salient unconditioned natural stimuli, independently of valence.

Thus, selective prefrontal NE depletion abolished the place conditioning induced by highly salient stimuli (i.e., WCh and IPL) in control animals and by mildly salient stimuli (i.e., MCh and IL) in stressed groups, but had no significant effects in control animals exposed to mild salient stimuli. These results demonstrate that prefrontal-accumbal CA transmission is necessary for the acquisition of conditioned properties to stimuli paired with highly salient natural rewarding or aversive events in a place-conditioning procedure. Many different factors have major regulatory role in motivated behaviors, including the internal variables of the organism (i.e., motivational state, stress response) and stimulus properties (i.e., salience or intensity), both of which affect motivational salience attribution processes (Berridge and Robinson, 1998; Richard and Berridge, 2011). It has been recently proposed that appetitive and aversive brain systems act in a "congruent manner for processes sensitive to affective intensity (salience) but not valence" (Belova et al., 2007), thus suggesting that a common neural system might be involved in processing stimuli salience, irrespectively of valence. Moreover, arousing pleasant or aversive stimuli that elicit valence-specific responses have been suggested to enhance attention and memory formation through a common, valence-insensitive pathway (Belova et al., 2007) and prefrontal cortex has been involved in processing both rewarding and aversive stimuli (Rolls, 2000; O'Doherty et al., 2001; Killgore et al., 2003; Ventura et al., 2007).

Dopaminergic transmission within NAc is considered to mediate the hedonic impact of reward or some aspects of reward learning (Everitt and Robbins, 2005 for review). Our results, in agreement with a different view (Berridge and Robinson, 1998), show that DA transmission in NAc plays a role in both positively and aversively motivated behavior; most importantly, however, they demonstrate that this motivational process is governed by prefrontal cortical NE.

Norepinephrine in mpFC might activate mesoaccumbens DA release through excitatory prefrontal cortical projection to VTA DA cells (Sesack and Pickel, 1992; Shi et al., 2000) and/or through corticoaccumbal glutamatergic projections (Darracq et al., 2001). Moreover, a role for mpFC projections to the LC in exerting an excitatory influence can be envisaged because this nucleus has been shown to activate VTA DA neurons (Grenhoff et al., 1993; Jodo et al., 1998; Liprando et al., 2004), which could lead to increased DA release in NAc. However, since amygdala is involved in Pavlovian conditioning of emotional responses and plays a specific role in modulating memory for arousing experiences (Balleine and Killcross, 2006; McGaugh, 2006), and given the complex anatomical and functional connections between this brain area and prefrontal cortex (Cardinal et al., 2002; Roozendaal et al., 2004), a role of the prefrontal cortex-amygdala system in the effects of the highly salient stimuli reported here must be considered (Belova et al., 2007; Mahler and Berridge, 2011).

Note that NAc and dopaminergic transmission are considered to play major role in motivation processes besides the role that DA plays in other aspects of incentive motivation and instrumental learning (Salamone et al., 2005). Indeed, based on the view that is doubtful that accumbens DA performs only one function, substantial evidences support the hypothesis that DA is involved in the exertion of effort or effort-related decision-making (Salamone et al., 2007; Bardgett et al., 2009), that is not incompatible with the involvement of this system in instrumental learning, incentive motivation, or pavlovian-instrumental transfer. Animal and human studies seem to converge in that, together with animal studies focused on effort related functions of accumbal DA, clinical findings are consistent with the hypothesis that DA systems are involved in behavioral activation, pointing to a striking similarity between the brain systems implicated in effort-related processes in animals and those involved in energy dysfunctions in humans (Salamone et al., 2007). According to this view NAc functioning is to be considered, along with prefrontal cortex and the amygdala, as a component of the brain circuitry regulating effort-related functions. In this framework, the prefrontal/accumbal CA system we have envisaged may conceivably be a part of a complex network involving cortical and subcortical brain areas involved in regulation of effort-related functions controlling motivation outcomes, and possibly linking salience intensity to effort intensity. In our view the impact of salient stimuli is crucial in the processes that lead to attribution of motivational salience, due to the salience perceived. That means that the impact of stimuli produce an emotional response that tunes the association processes leading to motivation outcome, thus pointing to the basic role of emotional salience as the individual is exposed to the UCS. The prefrontal/accumbal system we propose to control motivational salience processes depending on salience intensity should be considered included in complex networks regulating perceived emotion (Phillips et al., 2003b). Emotion perception, according to the appraisal theories (Arnold, 1960; Lazarus, 1991) has been suggested to stems from three processes: the identification of the emotional significance of a stimulus, the production of an affective state in response to the stimulus, and the regulation of the affective state. As shown by human and animal literature (Phillips et al., 2003b for review), these processes depend on different brain emotional systems involving brain areas including mesencephalic, cortical, and subcortical such as the amygdala, insula, ventral striatum, ventral, and dorsal anterior cingulate gyrus, the septo-hippocampus system, the prefrontal cortex, all characterized by reciprocal functional relationships

(Salzman and Fusi, 2010). The septo-hippocampus system has been considered as a general-purpose comparator, with a central role in determining the extent of conflict between different goal-directed behaviors (Gray and McNaughton, 2000). Amygdala has a well-known role in emotion and in memory consolidation processes depending on emotional arousal. Recently a role of this area in decision-making has been envisaged. Indeed, the amygdala can evoke conditioned responses capable of exerting a dominant effect on choice, and perceived emotional values in Pavlovian conditioning are exploited by instrumental (habit-based and goal-directed) learning mechanisms through connectivity with other brain regions such as the striatum and prefrontal cortex (Seymour and Dolan, 2008).

It is worth noting that "values" are affected by the action of stress hormones, such as glucocorticoids, on amygdala, and these effects control memory consolidation pointing to a link between emotional salience and the strength of memories (Roozendaal, 2000; Setlow et al., 2000; McGaugh, 2005). Moreover glucocorticoids have been shown to be biological substrate of reward (Piazza and Le Moal, 1997) and substantial evidences show that they play a role in the modulation of both appetitive and aversive emotional memories indicating that modulation of appetitive and aversive discrete-cue learning may be subserved by a common mechanism (Zorawski and Killcross, 2002).

We have provided evidence that prefrontal-accumbal CA system is involved in processing motivational salience attribution selectively when intense motivational salience is processed, thus pointing to an allegedly different neural system involved in the attribution of motivational salience related to mildly salient stimuli. Our results are consistent with those that had shown DA transmission is not always involved in motivation (Nader et al., 1997, for review). In contrast with the DA hypothesis that is based on a single-system model of reward, a non-deprived/deprived model has been proposed in the late nineties that claims that two separate neurobiological reward systems can be double dissociated, each of which makes a significant contribution to motivated behavior depending on deprivation state. Note that the model is supported by experiments where drug naive animals are considered similar to food-sated (i.e., non-deprived), differently from drug-dependent animals in withdrawal or food-restricted animals that are considered deprived (Nader et al., 1997; Laviolette et al., 2004). The model has two important implications. First, the relationship between the two systems appears to be mutually exclusive. A state of deprivation inhibits the non-deprived system [involving the peduncolo-pontine nucleus (TPP)]. Thus, the differential activation of the two systems is predicated specifically on whether animals are, for instance, in a state of withdrawal or not (Nader et al., 1997). The second implication is that a state of deprivation engages a second neurobiologically distinct motivational system, a component of which is DA.

The obvious question that arises from this model is whether all motivated behaviors can be considered to have a non-deprived and a deprived component. As it was questioned by the proponents (Nader et al., 1997, for review): "Do some stimuli only work through one of the two systems?" Although this discussion is out of the aim of our present work, we can't help noting the parallelism between our findings on prefrontal-accumbal CA system and the non-deprived/deprived system, in that our system is crucial in attribution of motivational salience when the stimuli salience is high and characterized by high emotional impact (either positive or negative). In this case, another system involved in low salience processing is inhibited or "off-line," and this system that is on-line when low salience is processed (and that we did not envisaged yet), parallels the non-deprived system, characterized by low emotional impact. Our results also suggest strongly that, like suggested for the non-deprived/deprived model, the system processing high salience (prefrontal-accumbal CA system) and the supposed one involved in low salience are mutually exclusive. In terms of the neural dynamics involved in the selective and exclusive engagement of these systems, we can tentatively pose that the graded increase of NE outflow in mpFC depending on low or high salience of stimuli, may involve different AR-subtypes, that, in turn, depending on a given threshold level of released NE, will engage different circuits, and, in the case of high salience, including DA in the NAc. This is the aim of ongoing experiments that will possibly elucidate this critical question.

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Interactions between epinephrine, ascending vagal fibers, and central noradrenergic systems in modulating memory for emotionally arousing events

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It is well-established that exposure to emotionally laden events initiates secretion of the arousal-related hormone epinephrine in the periphery. These neuroendocrine changes and the subsequent increase in peripheral physiological output play an integral role in modulating brain systems involved in memory formation. The impermeability of the blood brain barrier to epinephrine represents an important obstacle in understanding how peripheral hormones initiate neurochemical changes in the brain that lead to effective memory formation. This obstacle necessitated the identity of a putative pathway capable of conveying physiological changes produced by epinephrine to limbic structures that incorporate arousal and affect related information into memory. A major theme of the proposed studies is that ascending fibers of the vagus nerve may represent such a mechanism. This hypothesis was tested by evaluating the contribution of ascending vagal fibers in modulating memory for responses learned under behavioral conditions that produce emotional arousal by manipulating appetitive stimuli. A combination of electrophysiological recording of vagal afferent fibers and *in vivo* microdialysis was employed in a second study to simultaneously assess how elevations in peripheral levels of epinephrine affect vagal nerve discharge and the subsequent potentiation of norepinephrine release in the basolateral amygdala. The final study used double immunohistochemistry labeling of *c-fos* and dopamine beta hydroxylase (DBH), the enzyme for norepinephrine synthesis to determine if epinephrine administration alone or stimulation of the vagus nerve at an intensity identical to that which improved memory in Experiment 1 produces similar patterns of neuronal activity in brain areas involved in processing memory for emotional events. Findings emerging from this collection of studies establish the importance of ascending fibers of the vagus nerve as an essential pathway for conveying the peripheral consequences of physiological arousal on brain systems that encode new information into memory storage.

Keywords: amygdala, emotional arousal, epinephrine, learning, memory, vagus nerve

INTRODUCTION

An extensive number of findings reveal that the emotional nature of learning experiences contributes to the strength that novel events are encoded and stored into memory. The influence of highly arousing events on memory is attributed to the impact salient stimuli have in initiating and maintaining heightened levels of neural activity in the amygdala (Cahill and McGaugh, 1995; Gerra et al., 1996; Canli et al., 2002; Chang et al., 2005; Pelletier et al., 2005). Emotional experiences influence the amydala and regulate how effective new events are converted into memory by their capacity to evoke epinephrine secretion from the adrenal glands (McCarty and Gold, 1981) which in turn initiates a long lasting and sustained release of norepinephrine in the amygdala (Williams et al., 1998; O'Carroll et al., 1999; Hurlemann et al., 2005). The contribution of norepinephrine activation of the amygdala to memory processing is revealed by studies showing that infusion of this transmitter or beta-noradrenergic agonists into the basolateral amygdala selectively improve memory for responses acquired in inhibitory avoidance (Ferry et al., 1999), contextual fear conditioning (LaLumiere et al., 2003; Huff et al., 2006), or spatial learning tasks (Hatfield and McGaugh, 1999). Although a great deal of attention has been devoted to understanding how activation of the amygdala modulates neuronal functioning in other limbic structures during memory formation, less emphasis has been placed on identifying how changes in peripheral autonomic activity produced by adrenal hormones, feedback to the brain to influence noradrenergic activation of the amygdala during this important process.

Elucidation of the mechanisms underlying these interactions will provide a more comprehensive understanding of memory formation since they have circumscribed effects on how effective new events are represented in memory. For example, the uniform enhancement in memory observed following heightened states of arousal produced by epinephrine release is abolished by

experimental manipulations that disrupt amygdala functioning. Epinephrine's actions on memory are also ineffective when the amygdala is lesioned (Cahill and McGaugh, 1991) or if the major input and output pathways from the amygdala are severed (Liang et al., 1990; Roozendaal and McGaugh, 1996). Manipulations that produce more selective effects on neurotransmitter functioning such as blocking receptors that bind norepinephrine (Liang et al., 1986; Quirarte et al., 1997) or depleting amygdala norepinephrine concentrations with the selective neurotoxin DSP-4 (Liang et al., 1995) also block the memory enhancing actions of epinephrine. These two distinct neurochemical changes that follow emotional arousal have gained a great deal of attention for their role in modulating memory, however, the pathway that epinephrine secretion initiates and/or regulates a sustained increase in central noradrenergic output is not well understood. Such transmission is not likely to be a direct process because epinephrine does not gain access to the CNS due to the restrictive properties of the blood-brain barrier (BBB) (Weil-Malherbe et al., 1959; Bradbury, 1993).

In light of the restricted access of epinephrine to the brain, reports from anatomical, electrophysiological, and pharmacological experiments suggest that epinephrine's actions on memory and in potentiating norepinephrine output in the amygdala may be mediated by the initial activation of peripheral vagal fibers that project to the brain. The vagus nerve is considered a potential candidate to mediate the actions of epinephrine and by implication, emotional arousal on the brain because dorsal and ventral branches of the nerve innervate the adrenals (Coupland et al., 1989), electrical stimulation of the adrenal nerve, which results in epinephrine release evokes action potentials in the vagus (Niijima, 1992) and ascending fibers of the vagus are densely embedded with beta-adrenergic receptors that bind epinephrine (Schreurs et al., 1986; Lawrence et al., 1995). Blocking peripheral beta-adrenergic receptors abolishes arousal-induced changes in memory, peripheral autonomic functioning or epinephrine induced increases in vagal nerve firing (van den Buuse et al., 2001; van den Buuse, 2002; Carrive, 2006; Miyashita and Williams, 2006; King and Williams, 2009). The vagus nerve is also an ideal candidate to subserve this function because peripheral endings of the nerve innervate organs such as the heart and lungs that display heightened activity in humans that show enhanced retention in response to viewing emotionally arousing slides or animals displaying similar cognitive improvements following epinephrine administration (Shapiro and Miselis, 1985; Coupland et al., 1989; Paton, 1998a,b).

Information regarding changes in the functioning of the adrenals and other organs after exposure to arousing stimuli are transmitted via afferent fibers of the vagus nerve (Coupland et al., 1989; Paton, 1998a,b) to a region of the brainstem known as the nucleus tractus solitarius (NTS: Kalia and Mesulam, 1980; Sumal et al., 1983). The projection of vagal afferents to the NTS is important in understanding how vagal activation may affect distinct neuronal circuits to regulate norepinephrine release in the amygdala. For instance, ascending fibers of the vagus synapse on the A2 norepineprhine-containing neurons in the NTS (Sumal et al., 1983) that course through the brainstem to innervate and release norepinephrine in the amygdala (Fallon et al., 1978; Bjorklund and Lindvall, 1986; Riche et al., 1990; Petrov et al., 1993). A2 neurons in the NTS also project directly to locus coeruleus neurons (LC; Van Bockstaele et al., 1999) that provide the major source of noradrenergic innervation to the basolateral amygdala (Fallon et al., 1978). Interestingly, electrical stimulation of ascending vagal fibers or epinephrine injection alone, produces significant and long lasting increases in amygdala norepinephrine levels that are attenuated by inactivating the site of termination of vagal axons in the brainstem, the NTS (Williams et al., 1998, 2000; Hassert et al., 2004). Thus, vagal activation in response to epinephrine secretion may represent one mechanism by which emotionally arousing events facilitate memory processing by initiating norepinephrine release in the amygdala.

However, no findings to date have demonstrated simultaneously, that vagal nerve firing consequent to elevations in peripheral epinephrine levels, influence noradrenergic functioning in the amygdala to facilitate memory processing. The following studies were conducted to address this shortcoming with three separate approaches. These experiments examined this hypothesis by first determining if stimulating vagal afferent fibers at an intensity that augments norepinephrine release in the amygdala (Hassert et al., 2004), enhances memory for responses learned under behavioral conditions that produce emotional arousal by manipulating appetitive stimuli as opposed to administering noxious stimuli such as footshock. A second study utilized electrophysiological recording of vagal afferent fibers in conjunction with *in vivo* microdialysis to assess simultaneously, how elevations in peripheral levels of epinephrine affect vagal nerve discharge and the subsequent potentiation of norepinephrine release in the basolateral amygdala. The final study used double immunohistochemistry labeling of *c-fos* and dopamine beta hydroxylase (DBH), the enzyme for norepinephrine synthesis to determine if epinephrine administration alone or stimulation of the vagus nerve in separate groups of subjects produce similar patterns of neuronal activity in brain areas that process memory for emotional events. Findings emerging from this collection of studies are expected to reveal the contribution of ascending fibers of the vagus nerve in serving as an essential pathway for conveying the peripheral consequences of emotional arousal induced by epinephrine secretion on brain systems that encode new experiences into memory storage.

EXPERIMENT 1

The objectives of the first experiment were two-fold. This study determined if reducing the quantity of expected rewards produces emotional changes associated with frustration (i.e., the "Crespi Effect") that are manifested by decreases in motivation and behavioral responding. These changes are indexed by increased latencies to run the length of the runway to receive the reduced quantity of food rewards. The second goal was to determine whether retention of the emotional experience involving reward reduction is improved by increasing noradrenergic drive within the amygdala by post-training stimulation of vagal afferent fibers with an intensity that has been shown to produce significant and long lasting elevations in norepinephrine release in this structure.

METHODS

SUBJECTS

Twenty-three male Sprague-Dawley rats (275–300 g) obtained from Charles River Laboratories (Wilmington, MA) were used in Experiment 1. The rats were individually housed in polypropylene cages with corncob bedding and maintained on a standard 12:12 h light–dark cycle with lights on at 7:00 a.m. Food and water were available ad libitum during the seven day adaptation period to the vivarium.

SURGERY TO IMPLANT VAGAL STIMULATION ELECTRODES

Atropine sulfate (0.4 mg/kg) was given ip 10 min before anesthesia with sodium pentobarbital (50 mg/kg). Each rat was implanted with a stimulating electrode on the left vagus nerve at the cervical level that was constructed from two 30-gauge, 7 cm long insulated silver wires, stripped 1 mm on one end (the electrode end), and 1 mm on the other end (the current source end). As shown in **Figure 1**, polyvinyl chloride (PVC) tubes were placed over the 7 mm exposed section of each wire and contact was achieved by forming the electrode into a helix that, when implanted, would encircle the nerve. The two wires were inserted into a 3.5 cm piece of PVC and the electrode was completed by soldering metal female pins to the 1 mm stripped leads. The electrode leads from the vagus nerve were then drawn under the skin through the neck to the dorsal surface just posterior to the skull and affixed to a head post constructed from an Amphenol miniature strip connector and cemented to the skull with dental acrylic. The male pins were later attached to the female pins via 30 gauge silver wires to deliver electrical stimulation supplied by a current generator (Model 82400, Lafeyette Instruments, Lafeyette, IN). Electrode placement along the vagus was verified visually and by monitoring resistance between the two stimulating electrodes with a digital voltmeter. The resistance was constantly monitored to ensure the effectiveness of the vagus nerve stimulation (VNS).

STRAIGHT ALLEY FOOD REWARD TASK

Apparatus

As shown in **Figure 1**, the training apparatus consisted of a 72-inch long straight alley that was 15 inches in height, 10 inches wide and constructed of wood. The apparatus included a start compartment (6 inches long) that was blocked by a sliding door connected to a digital timer. The alley also included three hurdles (5 cm high) that were placed 2, 12, and 24 inches from the start box to ensure that a standard level of effort and motivation develops to obtain the food rewards. The goal cup containing the food rewards was 1 inch wide and placed at the end of the alley.

Training

Rats were placed on a weight maintenance schedule seven days before training to reduce weights to 15% below *ad-lib* feeding weights. Each animal was pre-exposed for two days to sucrose pellets (45 mg; Research Diets, Inc, New Brunswick, NJ) used for rewards throughout the study. Animals were randomly assigned to groups that would be given either sham or vagal stimulation following a *shift* or reduction in food reward from the normally expected 10 pellets to a small reward of only one sucrose pellet on Day 24. Training began by placing each rat in the start box facing away from the closed sliding door. The door was opened and the rat was given 120 s to jump over the three hurdles to reach and consume the sucrose pellet rewards placed in the food cup at the end of the six-foot long alley. As soon as the reward was consumed, the trial ended and the rat was placed in a holding

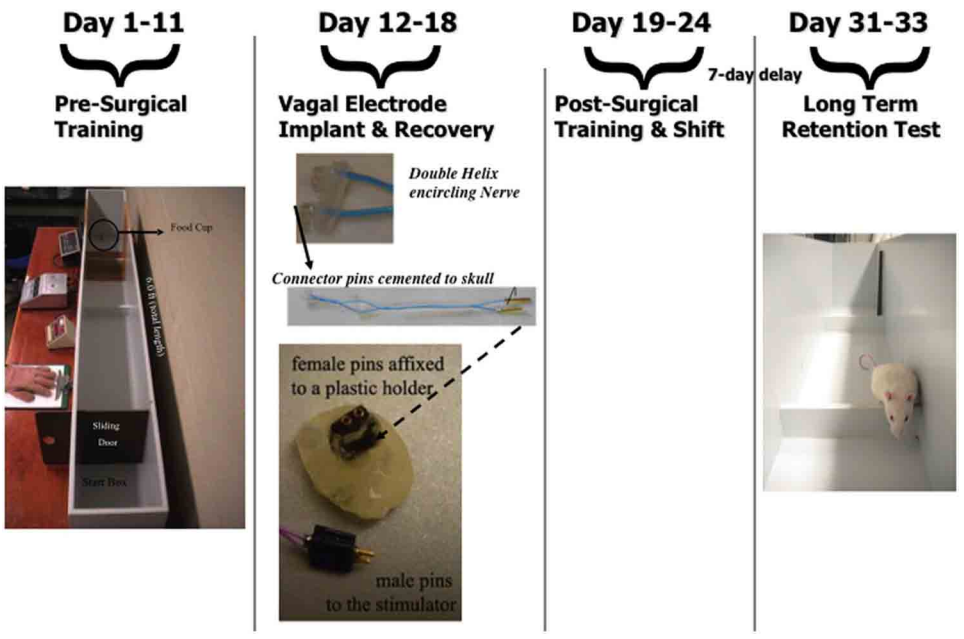


FIGURE 1 | Time line for the training and testing regimen in the straight alley learning task and dimensions of the training apparatus. Illustration of the electrode implanted along the vagus nerve to produce vagal nerve stimulation.

cage for an inter-trial interval (ITI) of 30 s. The latency to consume the food reward was measured on each of the four daily trials using a digital timer. The animals were trained on Days 1–11 prior to surgery (**Figure 2A**) to implant the vagal stimulating electrode. They were then given seven days of postoperative recovery (Days 12–18) before training resumed on Day 19 and continued for 5 days (See **Figure 1** for timeline).

Induction of emotional frustration by shifting reward magnitude

The experimental or "*Shift*" day in which emotional frustration was produced by the reduction in reward magnitude from 10 to 1 food pellets occurred on Day 24. On this day, animals assigned to the "*No-Shift*" group continued to receive 10 pellets while those in the "*Shift Group*" received only one, rather than 10 pellets on 6, as opposed to the normal four daily training trials. Each rat was removed from the alley after the sixth trial and those in the vagus nerve stimulation group received three, 10 s stimulations (0.4 mA current separated by 10 s intervals) along the left cervical vagus. The stimulation parameters were selected from those previously shown to produce long lasting elevations in amygdala concentrations of norepinephrine (Hassert et al., 2004) after VNS. Animals in the sham groups received no stimulation but were connected to the stimulating device. The four groups in Experiment 1 consisted of a *Sham-No Shift (Sham-NS), VNS-No Shift (VNS-NS), Sham-Shift, and VNS-Shift*. The subjects remained on the weight maintenance schedule and were not disturbed during the seven day interval between shift in reward magnitude and the first retention test (Days 24–31). This extended delay interval was imposed to examine whether memory of the frustrating experience of reward reduction is encoded more effectively by activating the vagus nerve following training since retention for this type of change in reward contingency decays in control animals after

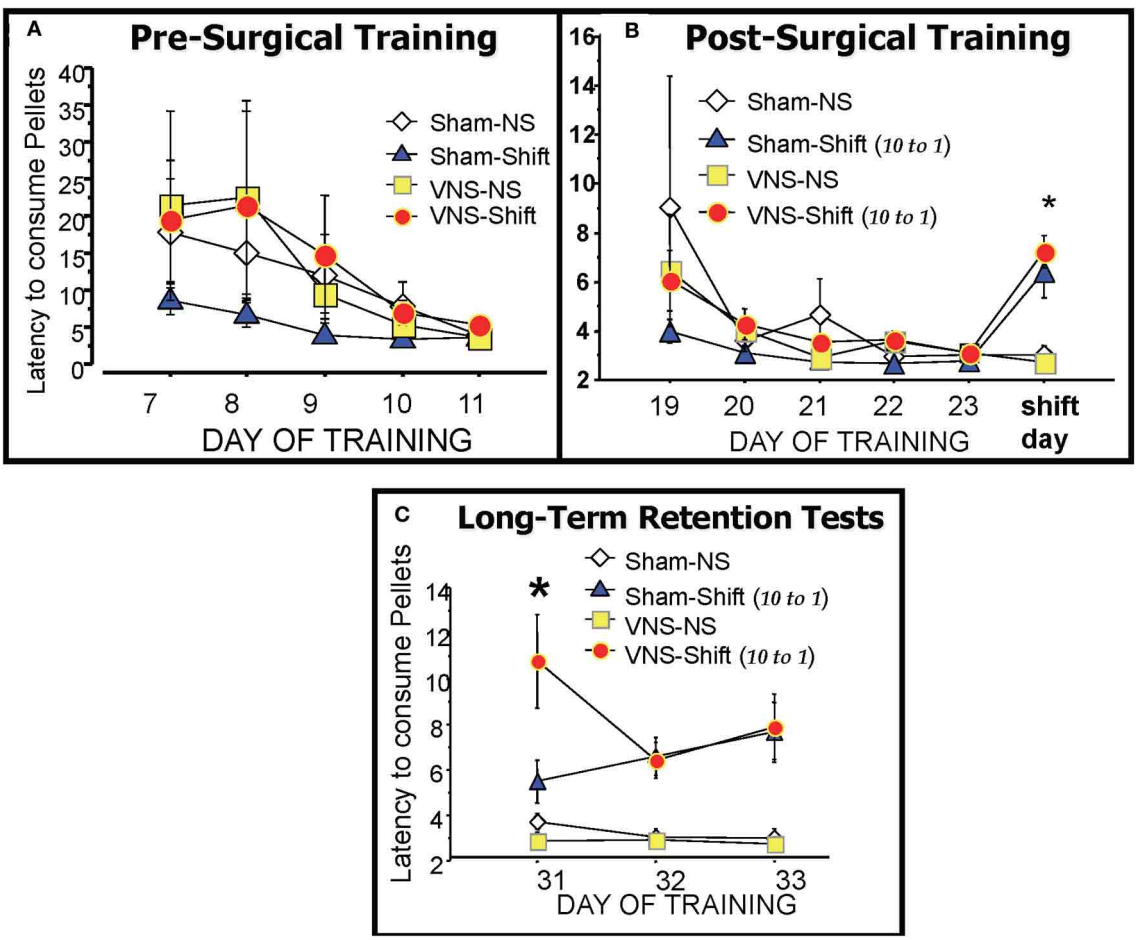


FIGURE 2 | The runway latencies of each training group during pre-surgical training (A), post-surgical training (B) and during the three days of retention testing (C). There were no significant differences between groups during the training periods. The reduction in reward magnitude from 10 pellets to only 1 pellet on the day of the *Shift* resulted in significantly longer latencies in the two shifted groups (Sham-Shift and VNS-Shift) relative to the *Non-Shifted* controls (Sham-NS and VNS-NS). The VNS-Shift group received vagal stimulation after the six training trials with the reduced reward whereas the Sham-Shift group was connected to the stimulator but no current was applied. Retention for the frustrating experience of reward reduction produced by the *Shift* was assessed one-week later on three daily retention tests. Animals in the vagal stimulation *Shift* group displayed enhanced retention on the 7 day delayed retention test as evidenced by their continued long latencies to traverse the maze to consume the reduced reward of 1 pellet. Details included in the text.

a one-week delay but remains intact in groups administered memory modulating treatments (Salinas et al., 1996).

Retention test

During the three days of retention testing (Days 31–33), animals in the shifted groups continued to receive a 1 pellet food reward on each of the six retention trials while those in the non-shift groups received the standard 10 pellets upon reaching the goal box. The latency to run the length of the alley and cross the three hurdles to consume the 10 pellets (non-shift groups) or the reduced quantity of one pellet (shifted groups) was recorded and served as an index of memory for the reduction in food reward.

Statistical analysis

Behavioral measures from the straight alley task are expressed as mean ± SE. A One-Way analysis of variance (ANOVA) followed by post hoc tests were used to detect differences in the latency to consume the food rewards between Shifted Sham and VNS groups as well as the No-Shift controls. Repeated measure ANOVA's were used to analyze differences in the latency to consume the reward between treatment groups during the training days, the shift day, and the first trial of the retention test.

RESULTS

EFFECTS OF REDUCING THE MAGNITUDE OF EXPECTED REWARDS

As depicted in **Figure 2B**, performance was statistically similar in all groups during the 5 days of post-operative training that preceded the shift in reward magnitude on Day 24. On this day however, decreasing the expected value in reward from 10 to only 1 pellet caused a significant overall change in the runway performance of the shifted groups as revealed by a One-Way ANOVA $F_{(3,\ 19)} = 9.137$, $p < 0.01$. $F_{(3,\ 21)} = 8.44$, $p < 0.01$. As shown in (**Figure 2B**), *Post-hoc* analysis revealed that the two shifted groups took significantly longer to cross the three hurdles and run the length of the six foot long alley to consume the new quantity of one reward pellet compared to the two non-shifted groups (Sham-Shift vs. Sham—NS, $p < 0.01$; Sham-Shift vs. VNS-NS, $p < 0.01$; VNS-Shift vs. Sham-NS, $p < 0.01$; VNS-Shift vs. VNS-NS, $p < 0.01$). These findings demonstrate the procedures used to induce emotional frustration through reward reduction produce reliable changes in behavior that are reflected in increased latencies and lower motivational levels to approach and consume the food rewards.

EFFECTS OF VNS ON LONG TERM RETENTION

A comparison of the mean runway latencies recorded during the six daily trials on the three days of retention testing is shown in **Figure 2C**. Post-training VNS produced a significant enhancement in memory for reward reduction on the first retention test given 7 days after the *Shift*. A One-Way ANOVA revealed a significant group difference [$F_{(3,\ 19)} = 9.575$; $p < 0.01$]. *Post-hoc* comparisons with Fisher's PLSD tests showed that the runway latencies of the VNS-shifted group were significantly longer than both non-shifted groups (VNS-Shift vs Sham-NS, $p < 0.01$; VNS-Shift vs. VNS-NS, $p < 0.01$) as well as the Sham-Shift group that also experienced a reward reduction ($p < 0.01$). The lasting consequences of reward reduction were evident on each of the three retention tests given on Days 31–33. A One-Way ANOVA on retention day 2 [$F_{(3,\ 19)} = 9.613$; $p < 0.01$] and 3 [$F_{(3,\ 19)} = 7.172$, $p < 0.01$] revealed that both the shifted groups exhibited longer latencies to consume the reward than the unshifted groups (Sham-Shift vs. Sham- NS, $p < 0.01$; Sham-Shift vs. VNS-NS, $p < 0.01$; VNS-Shift vs. Sham-NS, $p < 0.01$; VNS-Shift vs. VNS-NS, $p < 0.01$). The results demonstrate that increasing neural transmission from the vagus to the brain following an emotional event enhances memory and these effects are evident even when assessments of retention are delayed for as long as 7 days after the experience.

EXPERIMENT 2

Our previous findings demonstrated that neural activity recorded along the cervical vagus increased significantly following epinephrine injection and these excitatory actions were not observed in groups given an identical dose of the hormone after peripheral β-adrenergic receptor blockade with sotalol (Miyashita and Williams, 2006). These findings demonstrate that neural discharge in vagal afferent fibers is increased by elevations in peripheral concentrations of epinephrine and the effects are mediated in part by epinephrine binding to peripheral β-adrenergic receptors along the vagus nerve. This study also revealed the time course the nerve maintains high levels of firing following epinephrine coincides with the time course norepinephrine levels in the amygdala remain elevated after an identical dose epinephrine (Williams et al., 1998, 2000) or following stimulation of the vagus nerve at an intensity that improves memory (Hassert et al., 2004). When considered together, these findings raise a fundamental question as to whether neural impulses propagated along the vagus following emotional arousal and/or epinephrine secretion serve an important function in maintaining high-levels of noradrenergic transmission in the brain during the period of memory consolidation. However, no study to date has examined simultaneously, whether elevations in peripheral levels of epinephrine that lead to increased firing along ascending fibers of the vagus nerve, parallel the sustained changes in norepinephrine output that are observed in the amygdala. The present study combined electrophysiological and *in vivo* microdialysis approaches that were employed separately in the studies discussed above, to specifically test whether heightened activity in ascending vagal fibers following epinephrine administration regulates the release of norepinephrine in the amygdala.

METHODS

SUBJECTS

Forty-four male Sprague-Dawley rats purchased from Harlan Laboratories weighing approximately 250–300 g were used in this study. They were singly housed and maintained on a 12-h light-dark cycle (lights on at 6:00 a.m.) with water and food available *ad libitum*. The rats were handled daily for one week prior to the initiation of all experiments.

SURGERY

Two separate surgical procedures were performed in one-stage. The first involved severing the efferent rootlets of the left vagus nerve at the level of the cervix. This procedure was crucial

to insure that electrophysiological recordings in response to epinephrine administration derived solely from the intact ascending branches of the nerve and did not reflect impulses propagated along the cut descending fibers. The second procedure involved stereotaxic implantation of a microdialysis guide cannula above the left basolateral amygdala to measure extracellular concentrations of norepinephrine in response to epinephrine-induced changes in vagal nerve firing. The methods used to severe the efferent branch of the vagus nerve was adopted and modified from those of Norgren and Smith (1994). Each rat was anesthetized with an intramuscular injection of ketamine (100 mg/kg). A 3–4 cm incision was made from the chin to the thorax and the salivary gland, and lymph nodes were retracted laterally to expose the sternohyoid and omohyoid muscles. These two muscles were then separated from the underlying muscles to expose the superior laryngeal nerve and thyroid artery. A #15 blade in a scalpel handle was used to create an opening in the occipital bone to expose the efferent rootlets of the vagus nerve. The efferent rootlets were severed using ultra-fine forceps and the cavity was packed with bone wax. The retracted muscles, salivary gland, lymph nodes, and skin were repositioned and the skin was closed with suture.

Following deafferentation, each rat was placed in a stereotaxic apparatus and a midline incision on the skull was made to expose bregma. A single microdialysis guide cannula was lowered 2 mm above the basolateral amygdala (AP −3.0, L +5.0, DV −6.7) according to the atlas of Paxinos and Watson (1986). Following implantation, the guide cannula was cemented to the skull by two anchoring screws using dental acrylic. Animals were then allowed seven days to recover from both surgeries prior to electrophysiological recordings combined with *in vivo* microdialysis collection of norepinephrine.

Simultaneous vagal recording and microdialysis sample collection

On the experiment day, the rat was anesthetized with urethane (1.2 g/kg, i.p.) and a 4 cm incision was made 5 mm lateral to of the midline of the cervix to identify the internal carotid artery and cervical branch of the vagus nerve. Approximately 1 cm of the nerve was separated from connective tissue and placed onto two 30 gauge silver wire hook electrodes connected to a recording device. After the surgical preparation and recording settings were established, a 1:1 petroleum jelly and mineral oil mixture was used to cover the vagus nerve and electrodes, preventing dryness and the interference of tissue fluids during recording. The animal was left undisturbed for a 45 min period of nerve stabilization before any recordings were measured.

During recording, multifiber neural activity in the form of mV was collected from the electrodes, amplified 10–20,000 times using AC amplifiers (Grass, P511; Astromed: West Warwick, RI) with low (100 Hz) and high (1 kHz) frequency cutoffs. The signals were relayed to a spike processor to separate signals generated from action potentials from those with variable amplitudes derived from background noise. The window discriminator was set above the level of noise on a computer screen and the output of the spike processor representing action potentials from only the nerve fiber were averaged using the Chart data acquisition software (ADI instrument: Mountain view, CA) and monitored with an audio amplifier. For data analysis, the amplified signals were passed through an integrator with a time constant of 1 s and the summated electrical activity from action potentials recorded from the whole-nerve was stored on a PC computer and analyzed with the Chart program.

Concomitant microdialysis collection and electrophysiological recording from the vagus nerve

During the first hour pre-collection period, a microdialysis probe (CMA/12; Carnegie /Medicin, Acton, MA) was inserted into the left basolateral amygdala. After the vagal surgical procedures were carried out, the rat was left undisturbed until the pre-collection period was over. During this period, no brain samples or nerve activity was recorded. At the start of the second hour, dialysate samples of norepinephrine were collected every 20 min over a period of 60 min. The concentration of norepinephrine in these three samples was averaged to represent baseline levels of this transmitter in the amygdala. Electrical recordings of vagal basal activity were initiated during the final 30 min of this period and consisted of 15 minutes of baseline collection that was followed by i.p. injection of saline or sotalol to block peripheral adrenergic receptors in some groups and a second period of 15 min of collection to ensure the drug injections alone, did not affect vagal nerve firing.

The rats were randomly assigned to one of seven treatment groups. Four of these groups received an ip injection 15 min prior to the start of the experiment of either saline, 4.0 mg/kg of the peripherally acting beta-adrenergic antagonist Sotalol or 2.0 mg/kg of this antagonist (two groups). These groups would also be given an ip injection at the very beginning of the experiment at time period (0) of saline or epinephrine. This combination of treatments resulted in the following four groups (SAL, $n = 7$); sotalol 2.0 mg/kg + saline (SOT2 + SAL, $n = 6$); sotalol 4.0 mg/kg + saline (SOT4 + SAL, $n = 4$); sotalol 2.0 mg/kg + epinephrine 0.3 mg/kg (SOT2 + EPI, $n = 4$). Three additional groups received initial injections of 0.3 mg/kg of epinephrine at time period (0) and a second injection of 2.0 or 4.0 mg/kg of sotalol that was delayed by 60 min. These groups were included to determine whether blocking peripheral adrenergic receptors after vagal firing had reached asymptote attenuates neural impulses recorded from the vagus nerve. The groups consisted of epinephrine 0.3 mg/kg alone (EPI, $n = 6$); epinephrine 0.3 mg/kg + sotalol 2.0 mg/kg (EPI + SOT2, $n = 6$); and epinephrine 0.3 mg/kg + sotalol 4.0 mg/kg (EPI + SOT4, $n = 6$). The recording period lasted for 3 h after the first injection.

Norepinephrine assay with HPLC

Dialysate samples (35 ul) of norepinephrine were assayed by an HPLC system with a Waters 510 pump, Waters 717 autosampler, Atlantis T3 column (3 micron ODS, 4.6 × 100 mm) and a Waters 2465 electrochemical detector. The mobile phase consisted of 50 mg disodium EDTA, 13.8 mg monobasic sodium phosphate, and 58 mg octane sulfonate adjusted to pH 3.2 by adding 85% phosphoric acid. The flow rate was adjusted to 1.0 ml per minute.

Histology

The rat was deeply anesthetized with Euthosol (Virbac) and perfused with 0.9% saline and 10% buffered Formalin intracardially.

The perfused brains were stored in 10% buffered Formalin, sectioned at the thickness of 50 um using a Vibratome, mounted onto gelatin-coated slides, stained with cresyl violet, coverslipped, and then dried. The cannula tracts were verified using a microscope Olympus CX41. Three animals were excluded from the study due to incorrect cannula placement and electrophysiological data from two animals were discarded due to technical problems. This yielded 6, 6, 6, 7, 4, 4, and 6 animals in the EPI, EPI + SOT2, EPI + SOT4, SAL, SAL + SOT4, SOT2 + EPI, and SOT2 + SAL groups respectively for electrophysiological recording.

Statistical analysis

Electrophysiology. Two separate sets of analyses were performed to characterize the data collected from the vagal recording. Repeated measure and factorial ANOVAs were used respectively, to assess fluctuations in vagal neural activity across and within treatment groups during the 15 min drug free baseline period. Data points in the analysis were taken by averaging electrical activity of the vagus nerve in millivolts in 5 min bins. Any change in electrical activity measured in each subsequent 5 min bin during the 15 min drug free baseline period is expressed as the percentage change relative to this value. A repeated measure ANOVA was used to detect changes in whole nerve neural activity across the 180 min collection period. Factorial ANOVA's with *post-hoc* Fisher's tests were used to detect differences between individual treatment groups (i.e., SAL, SOT2 + SAL, SOT2 + EPI, EPI, SAL + SOT4, EPI + SOT2 EPI + SOT4). A level of $p < 0.05$ was deemed significant in all analysis.

In vivo microdialysis. The levels of norepinephrine (in picograms per microliter) collected from the basolateral amygdala during the initial 60 min baseline period was averaged to yield a standard baseline value of 100%. The concentration of norepinephrine collected from dialysate samples throughout the experiment is expressed as the percent change above or below the baseline value. A Two-Way ANOVA was used to evaluate the overall effect of treatment for each group relative to baseline levels. This resulted in a mixed factorial analysis with one between-subjects factor (treatments), one within-subjects factor (time), and one interaction (Treatments × Time).

RESULTS

VAGAL RECORDING

Baseline comparisons

There were no between group differences in vagal neural activity during the 15 min baseline period or the 15 min period that followed saline or sotalol injection. This tendency was consistent within and across each of the experimental groups. A Two-Way repeated measure ANOVA for changes in vagal neural acitivity during the 15 min drug free baseline period revealed no significant overall effects for treatment [$F_{(6, 32)} = 0.37$, $p = 0.89$] or time [$F_{(2, 64)} = 1.42$, $p = 0.24$] or interaction between time and treatment [$F_{(12, 64)} = 0.36$, $p = 0.97$]. Additional factorial ANOVAs used to detect overall differences in neural activity between the seven treatment groups during each of the three 5 min bins following saline or sotalol administration also yielded no significant effects.

Post-injection within group comparisons

Figure 3 shows that over the 3 h recording period following drug treatment, groups given epinephrine injection at time period (0) and sotalol 60 min later (i.e., EPI, EPI + SOT2, and EPI + SOT4) exhibited increases in vagal firing discharge that remained statistically significant above baseline during the 10–60 min post-epinephrine injection. For the remaining time of recording, vagal activity in the EPI and EPI + SOT2 groups gradually decreased to approximately 20% above the values measured at baseline. In contrast, vagal activity in the EPI + SOT4 group dropped steeply at minute 90 (*sotalol was given at 60 min after epinephrine injection*), and was reduced by 50% relative to baseline at the end of recording. In addition, neural activity recorded from the vagus nerve in the SAL, SAL + SOT4, SOT2 + EPI, and SOT2 + SAL groups showed a tendency to decrease over time with a reduction rate of 50% relative to baseline at the end. A Two-Way repeated measure ANOVA indicated that drug treatment caused a main group difference [$F_{(6, 32)} = 10.21$, $p < 0.0001$]. Additionally, time was another factor that contributed to the overall change in vagal neural activity [$F_{(19, 608)} = 42.28$, $p < 0.0001$]. An interaction between treatment drug and time was also observed [$F_{(114, 608)} = 2.47$, $p < 0.0001$].

Individual repeated measure ANOVAs comparing baseline levels of vagal activity with that occurring during the post-injection period also revealed significant differences across time in all seven treatment groups [EPI: $F_{(5, 19)} = 7.9$, $p < 0.0001$, EPI + SOT2: $F_{(5, 19)} = 3.6$, $p < 0.0001$, EPI + SOT4: $F_{(5, 19)} = 18.2$, $p < 0.0001$, SAL: $F_{(6, 19)} = 5.9$, $p < 0.0001$, SOT2 + SAL: $F_{(5, 19)} = 8.3$, $p < 0.0001$, SAL + SOT4: $F_{(3, 19)} = 5.1$, $p < 0.0001$, and SOT2 + EPI: $F_{(3, 19)} = 13.0$, $p < 0.0001$]. With the exception of the EPI, EPI + SOT2, and EPI + SOT4 groups, the significant changes identified in this analysis were attributed to reductions in neural activity recorded from this preparation during the 180 min post-injection period relative to baseline levels. In contrast, *post-hoc* analysis revealed that neural activity recorded from the vagus of animals given EPI, EPI + SOT2, EPI + SOT4 increased significantly above baseline values 10 min post-injection ($p < 0.05$) and remained significantly elevated for each of the next five periods of data collection (i.e., from 10 to 60 min).

Between group comparisons

From 10 to 60 min post epinephrine injection, neural activity along vagal ascending fibers were significantly higher in the EPI, EPI + SOT2, and EPI + SOT4 groups than any of the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups. A factorial ANOVA with *post-hoc* Fisher's tests run at each post-injection data collection point revealed significantly higher levels of vagal activity in the EPI, EPI + SOT2, EPI + SOT4 relative to groups treated with SAL, SOT2 + SAL, SOT2 + EPI, or SAL + SOT4 at each of the data collection points 10–60 minutes post-injection [10 min: $F_{(6, 32)} = 8.01$, $p < 0.0001$; 20 min: $F_{(6, 32)} = 8.11$, $p < 0.0001$; 30 min: $F_{(6, 32)} = 9.43$, $p < 0.0001$; 40 min: $F_{(6, 32)} = 12.70$, $p < 0.0001$; 50 min: $F_{(6, 32)} = 11.32$, $p < 0.0001$; 60 min: $F_{(6, 32)} = 9.58$, $p < 0.0001$; for *post-hoc* comparisons between

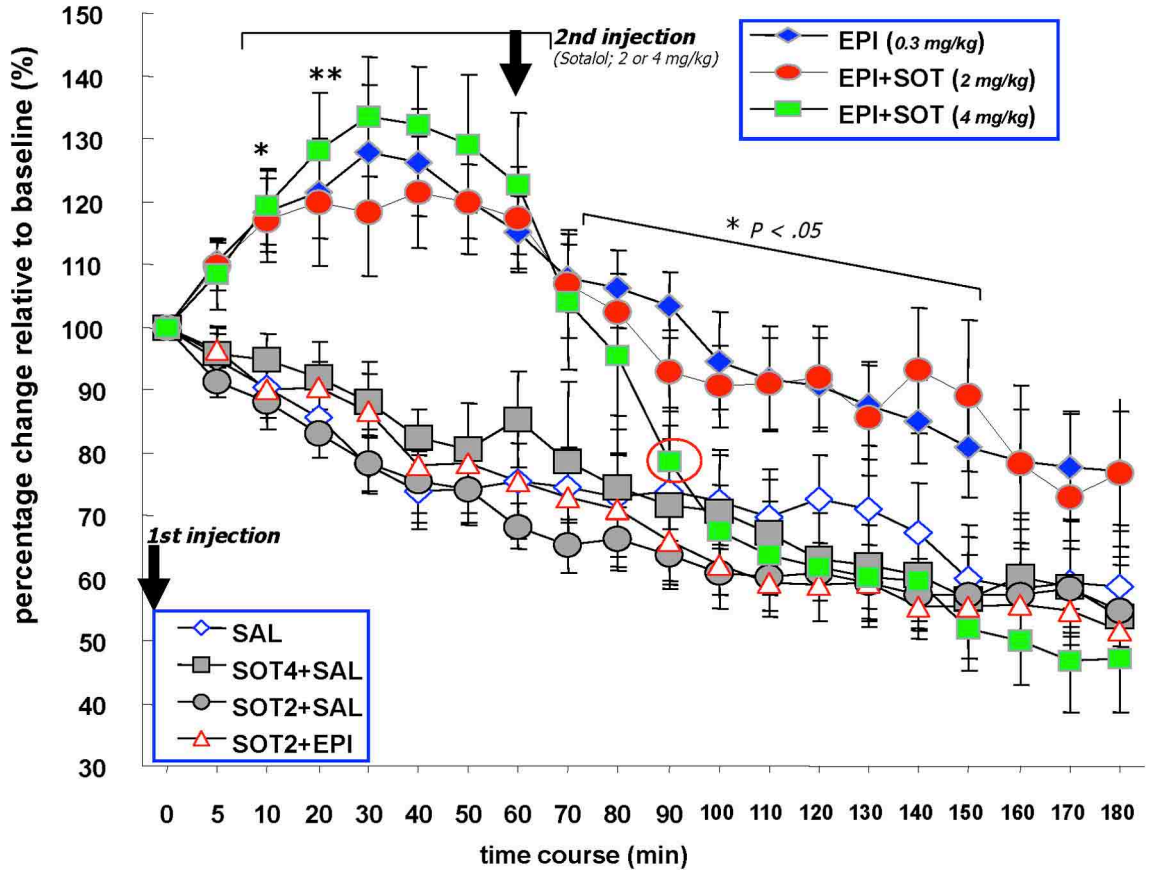


FIGURE 3 | The effects of systemically administered saline, epinephrine (0.3 mg/kg), sotalol (2.0 or 4.0 mg/kg) or the combination of epinephrine and sotalol on vagal nerve firing in Millivolts. Animals received sotalol 15 min prior to minute (0) and epinephrine at this time point. The first injection at 0 min. Two of the epinephrine injected groups received a second injection at 60 min that consisted of sotalol at 2 mg/kg or 4 mg/kg. One of the saline injected groups received a second injection at 60 min of sotalol at 4 mg/kg. The EPI, EPI + SOT2, and EPI + SOT4 groups exhibited significant increases in vagal firing discharge above baseline for 10–60 min post-epinephrine injection. For the remaining time of recording, vagal activity in the EPI and EPI + SOT2 groups gradually decreased to approximately 20% above basal values. In contrast, vagal activity in the EPI + SOT4 group dropped steeply at 40 min post-sotalol injectionand was reduced by approximately 50% relative to baseline at the end of recording. Neural activity recorded in the form of Millivolts from the vagus nerve in the SAL, SAL + SOT4, SOT2 + EPI, and SOT2 + SAL groups decreased over time with a reduction of approximately 50% relative to baseline at the end.

any of EPI, EPI + SOT2, and EPI + SOT4 groups vs. any of SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups, p-values < 0.05].

Factorial ANOVAs at 80–150 min post-injection continued to reveal significant overall differences between treatment groups. *Post-hoc* tests indicated that during this period, the EPI group continued to show a significantly higher-level of vagal activity than any of the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups ($p < 0.05$). During 110–150 min post-injection, vagal discharge of the EPI group remained higher than the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups ($p < 0.1$). For the remaining recording period (i.e., 160–180 min), vagal activity in the EPI group was not different from any of the control groups.

During 70–80 min post epinephrine injection, vagal activity of the EPI + SOT4 group was not different from the EPI and EPI + SOT2 groups. During 90–150 min post-epinephrine injection, the EPI + SOT4 group showed significantly lower vagal activity compared to the EPI group (EPI vs. EPI + SOT4, $p < 0.05$). During 90–180 min post-epinephrine injection, the EPI + SOT4 exhibited a level of vagal activity that was not different from that of any of the control groups, i.e., SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 ($p > 0.1$).

Norepinephrine release in the basolateral amygdala

Baseline comparisons. As shown in **Figure 4**, the baseline levels of norepinephrine were similar across treatment groups during the 60 min drug free baseline period. A Two-Way repeated measure ANOVA for changes in norepinephrine release for the 60 min baseline period showed no main effect by group [$F_{(6, 31)} = 0.1$, $p = 0.99$], time [$F_{(2, 62)} = 0.4$, $p = 0.66$], or their interaction [$F_{(12, 62)} = 0.3$, $p = 0.99$]. Individual factorial ANOVAs at each baseline collection point did not detect any significant group effect during the drug free period.

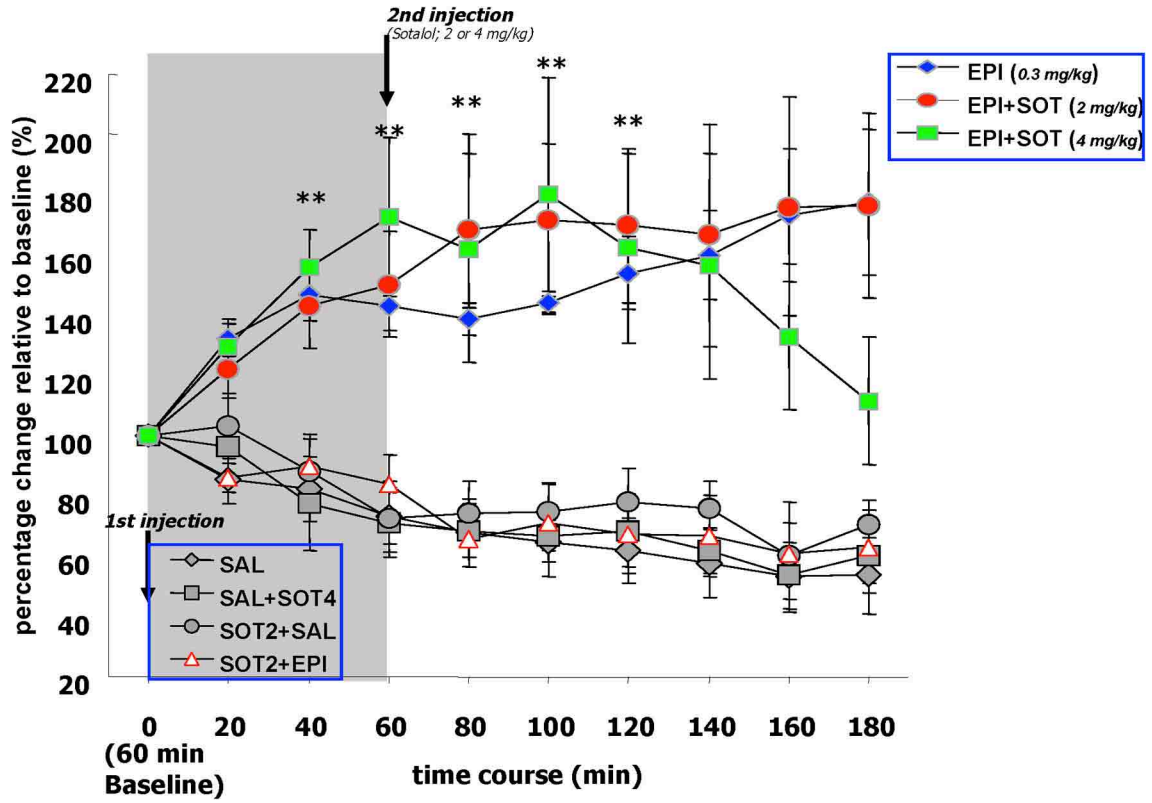


FIGURE 4 | The effects of systemically administered saline, epinephrine (0.3 mg/kg), sotalol (2.0 or 4.0 mg/kg), or the combination of epinephrine and sotalol on norepinephrine release in the amygdala. Basal levels of norepinephrine were similar across treatment groups during the 60 min drug-free baseline period. Norepinephrine concentrations sampled from the amygdala of the EPI and EPI + SOT2 groups showed consistent and sustained increases throughout the collection period whereas the norepinephrine levels of EPI + SOT4 became comparable to control groups at 180 min post-epinephrine injection. Blocking peripheral beta-adrenergic receptors with sotalol blocked epinephrine induced augmentation of norepinephrine output in the amygdala.

Post-injection comparaisons. During the 3 h post-epinephrine collection period, norepinephrine levels in the basolateral amygdala fluctuated differently depending upon a given drug treatment. Norepinephrine concentrations sampled from the amygdala of the EPI, EPI + SOT2 and EPI + SOT4 groups showed significantly consistent and sustained increases throughout the collection period whereas the levels sampled from the control groups, (i.e., SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4) showed reductions over time that were approximately 40% lower than the baseline values. A Two-Way repeated measure ANOVA comparing norepinephrine levels over the 180 min duration indicated significant differences between the seven treatment groups [$F_{(6,\ 31)} = 9.65$, $p < 0.0001$] as well as an interaction between treatment and time [$F_{(54,\ 279)} = 3.1$, $p < 0.0001$].

Individual repeated measure ANOVAs comparing norepinephrine levels at each post-injection collection time point with baseline values detected that there were increases of norepinephrine release over time in the EPI, EPI + SOT2, and EPI + SOT4 groups but decreases over time in the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups [EPI: $F_{(9,\ 45)} = 3.9$, $p < 0.001$; EPI + SOT2: $F_{(9,\ 36)} = 3.0$, $p < 0.01$; SAL: $F_{(9,\ 54)} = 8.6$, $p < 0.0001$; SOT2 + EPI: $F_{(9,\ 45)} = 3.90$, $p < 0.01$; SOT2 + SAL: $F_{(9,\ 27)} = 2.4$, $p < 0.05$; SAL + SOT4: $F_{(9,\ 27)} = 3.8$, $p < 0.01$]. *Post-hoc* analysis revealed that norepinephrine release in the EPI, and EPI + SOT2 groups increased significantly above initial baseline values at 20 min post-injection ($p < 0.05$) and remained significantly elevated for each of the following eight periods of data collection (i.e., from 40 to 180 min) ($p < 0.05$). In addition, significant changes identified in the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups were attributed to reductions in norepinephrine release during the 180 min post-injection period relative to baseline levels.

Between group comparison. Factorial ANOVAs detected group difference at each collection time point after epinephrine injection. *Post-hoc* tests comparing the EPI groups with the controls groups at each post-epinephrine collection point, revealed that levels of norepinephrine sampled from the amygdala were significantly higher than those of the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups (all p-values < 0.05). The EPI + SOT2 groups showed significantly higher levels of amygdala norepinephrine than any control groups over the 180 min post-epinephrine injection (i.e., $p < 0.05$) vs. SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4. The EPI + SOT4 group

exhibited significantly higher norepinephrine levels up to 140 min post-epinephrine injection and then declined for the remaining two periods of collection. At 180 min post-injection, the norepinephrine levels of the EPI + SOT4 group were significantly lower than those of the EPI and EPI + SOT2 groups. *Post-hoc* tests comparing the EPI + SOT4 with other groups at each post-epinephrine collection point, revealed that the levels of norepinephrine release in the amygdala was significantly higher than the SAL, SOT2 + SAL, SOT2 + EPI, and SAL + SOT4 groups during 40–140 min post-epinephrine injection (all p-values < 0.05). After 160 minutes, norepinephrine levels in the EPI + SOT4 group declined and was not different from any of the control groups ($p = 0.11$). *Post-hoc* tests revealed no significant difference in norepinephrine levels between any control group at each of the 180 min time periods. Additionally, norepinephrine release in these groups was reduced by approximately 40% at the end of collection.

EXPERIMENT 3

The capacity for emotionally arousing events to affect central structures that process memory may require the integration of peripheral epinephrine secretion, vagal neural transmission, activation of noradrenergic neurons in the brainstem, and noradrenergic release in important limbic structures. The current study will identify which components of this system are influenced by the two mechanisms that are known to be the peripheral substrates (i.e., increased epinephrine levels and vagal firing discharge) of an arousal related system that is involved in the modulation of memory. The patterns of activation induced by either epinephrine administration or VNS will be assessed using Fos immunocytochemistry. Additionally, activation of noradrenergic cells will be examined using Fos coupled with DBH immunocytochemistry. The immediate early gene c-fos is known to play a role in transcriptional regulation that leads to molecular changes for hours to days (Chiasson et al., 1997). At cellular levels, the expression of Fos protein is rapidly induced by the activity of neurotransmitters or neuromodulators, and electrical excitation at the cell surface (Morgan and Curran, 1986). Therefore, the detection of Fos protein is often used as a neuronal maker to label cells with activity changes in response to exogenous stimuli.

Findings from the current study will reveal whether or not increased levels of epinephrine and VNS produce similar patterns of neuronal activation within memory associated areas of the brain. Furthermore, localization of activated noradrenergic neurons with those that express Fos protein will reveal the contribution of noradrenergic nuclei in driving limbic areas that respond to peripheral physiological arousal. The current study would be the first to provide a comprehensive assessment on activation patterns in memory associated structures induced by different peripheral arousal mechanisms. If the patterns of activation following the two treatments are convergent, the findings will suggest that up-regulated peripheral adrenergic activity induced by emotional arousal increases neural transmission along ascending vagal fibers that in turn, generates cellular activity within central noradrenergic systems that modulate memory formation.

METHODS

SUBJECTS

Thirty-one male Sprague-Dawley rats purchased from Charles River Laboratories weighing approximately 250–300 g were used in this study. They were singly housed, and maintained on a 12-h light–dark cycle (lights on at 6:00 a.m.) with water and food available *ad libitum*.

EXPERIMENTAL TREATMENTS

Surgery

The procedures described in Experiment 1 to implant stimulation electrodes along the left cervical vagus were also followed for this study. After a one-week period of postoperative recovery, animals implanted with a stimulation electrode or unoperated controls were handled for 5 min on six consecutive days. On the 7th day of handling, animals in the VNS groups were stimulated in the handling room with an intensity of either 0.0 mA (sham) or 0.4 mA current (10 s on/off, six times) by connecting the vagal electrode with wires to a current source. The 0.4 mA current was selected because this level of intensity has been shown to improve retention performance in behavioral learning tasks (Clark et al., 1998, 1999). Animals in the epinephrine injection groups were handled in an identical manner but were given saline, 0.1 mg/kg, or 0.5 mg/kg epinephrine intraperitoneally. The doses were selected from those that have been shown to affect plasma epinephrine levels (McCarty and Gold, 1981) and memory performance (Gold and van Buskirk, 1975; Williams and McGaugh, 1993; Clayton and Williams, 2000).

Immunohistochemistry

Ninety minutes after VNS or epinephrine injection, each rat was deeply anesthetized with 1.3 ml/kg pentobarbital (Nembutal) and then perfused with 100 ml 0.01 M phosphate buffer saline (PBS) and then 2000 ml fresh 4% paraformaldehyde. The brain was then removed, post-fixed overnight at 4°C, stored in 30% sucrose sliced at the thickness of 50 um and stored in 0.1 M PBS with 0.1% sodium azide till the processing of immunostaining. Free floating immunostaining was started with 30 min of blocking endogenous hydrogen peroxidase by 0.3% H_2O_2 and 0.1% NaN_3 in 0.01 M PBS. After rinses, sections were then incubated in solution with 1:1000 goat anti-rat, 1% normal goat serum (NGS), 0.5% Triton X100, 0.1% NaN_3 in 0.01 M PBS for two hours at room temperature. After rinses, sections were then incubated in the primary antibody, 1:50,000 polyclonal rabbit anti-fos (ab5, Oncogene), with 1% NGS 0.5% Triton X100, 0.1% NaN_3 in 0.01 M PBS for 48 h at room temperature. After rinses, the sections were incubated in solution with 1:1000 goat anti-rabbit, 1% NGS, 0.5% Triton X100, 0.1% NaN_3 in 0.01 M PBS for overnight at room temperature. After rinses, sections were incubated with 1:500 avidin-biotin-peroxidase complex (ABC kit, Vector Laboratories) in 0.5% Triton X100 in 0.01 M PBS for 3–4 h at room temperature. After rinses in 0.05 M Tris buffer, all sections were simultaneously stained in 0.02% nickel-DAB (diaminobenzidine), 0.04% ammonium chloride sulfate, 0.15% nickel ammonium sulfate, 0.25% 3% H_2O_2 in Tris buffer for 5–10 min till the desired color, dark purple, developed. The reaction was stopped by rinsing the sections in 0.01 M PBS.

A second set of sections were used for double immunostaining of Fos and DBH. For the DBH immunostaining, the sections were processed as described above except for the primary antibody, mouse anti-DBH (Chemicon), for 36–48 h (1:30,000 mouse anti-DBH, 1% NGS, 0.5% Triton X100, 0.1% NaN_3 in 0.01 M PBS), and the DAB staining [0.02% DAB (diaminobenzidine), 0.04% ammonium chloride sulfate, 0.25% 3% H_2O_2 in Tris buffer] for 5–10 min until the desired brown color developed. After the completion of staining, sections were mounted on gelatin coated slides, dried overnight, cleaned in 75%, 95%, 100%, 100% ethanol, and Histoclear for 5 min in each solution, and then coverslipped with DPX.

Fos quantification

The number of Fos labeled cells were quantified in six brain regions, including the shell region of the NAC, CEA, BLA, HIP (dorsal portion), LC, VLM, and NTS. Additionally, the hypoglossal nucleus and the core region of NAC were selected as control areas to ensure that Fos was not expressed nonspecifically. Images were captured using an Olympus CX41 microscope at the magnification of 40X and a Nikon Coolpix 4500 digital camera. The quantification was conducted using the Scion Image program. To obtain representative estimates of Fos counts, each image underwent standard procedures using the Scion Image features described as follows: (1) defining the boundary of each brain region with reference to the Brain Atlas (Paxinos and Watson, 1997), (2) background subtraction to equalize the brightness and contrast, (3) computing Fos positive cells in the defined area using Scion Image tools. Fos counts in each brain region were obtained from a fixed number of coronal levels, **Figure 5** illustrates the specific levels counted in each of the examined brain regions.

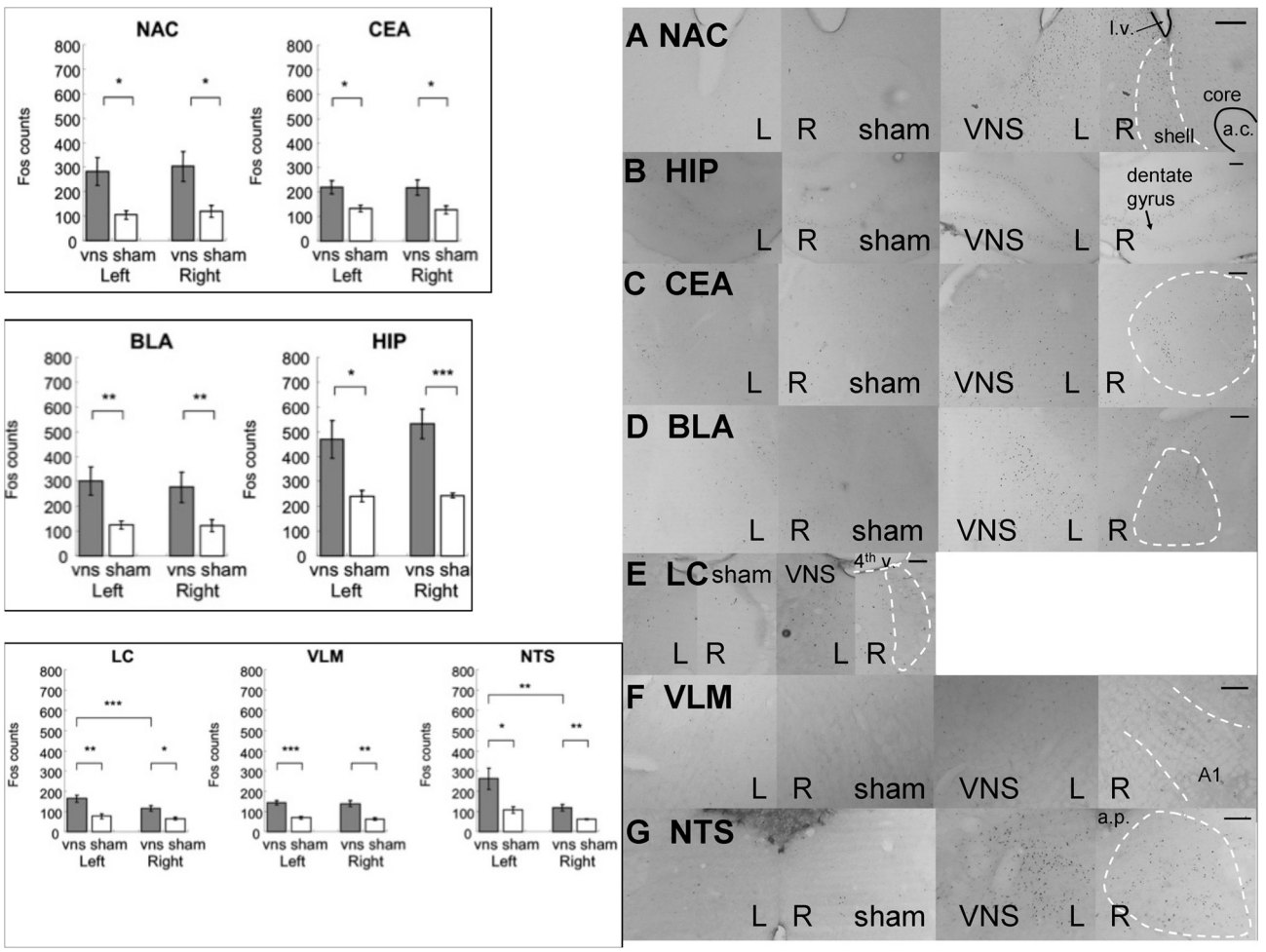


FIGURE 5 | Electrical stimulation of the left vagus nerve significantly increased Fos-like immunoreactivity in each of the examined brain regions bilaterally: NAC, CEA, BLA, HIP, LC, VLM and NTS. In addition, the stimulation also induced an ipsilateral predominance of Fos expression in the LC and NTS. (*, **, and *** denotes significant difference between the compared groups with a *p*-value less than 0.05, 0.01, and 0.001, respectively). Photomicrographs of Fos labeling in the NAC (A), HIP (B), CEA (C), BLA (D), LC (E), VLM (F), and NTS (G) following sham or vagus nerve stimulation. Scale bar = 200 um. (l.v.: lateral ventricle; a.c.: anterior commisure; shell: the shell region of the nucleus accumbens; core: the core region of the nucleus accumbens; 4th v.: the fourth ventricle; a.p.: area postrema.

Double labeled (Fos+ and DBH +) cells quantification

The number of double labeled cells was quantified in three brain regions including LC, VLM, and NTS. An Olympus CX41 microscope was used to visualize and quantify double labeled cells. Two criteria were developed to designate a given neuron as a double labeled cell: (1) a solid, purple/black nucleus and (2) brown cytoplasm around the nucleus. During quantification, brightness, and contrast of each image was adjusted to an optimal level. The observer was blind to the treatment condition.

Statistical analysis

Individual ANOVAs followed by Fisher's post hoc tests were used to compare Fos expression between the treatment and control groups within each brain region following VNS or epinephrine treatment. Because stimulation of the vagus nerve was unilateral, Two-Way repeated measure ANOVAs using treatment (sham or 0.4 mA) as the between subjects factor and hemispheres (ipsilateral or contralateral to vagal stimulation) as the within subjects factor were used to analyze data from the vagus nerve treatment. For epinephrine groups, One-Way ANOVAs were used to determine differences between the two epinephrine doses and saline. Statistical significance was achieved when a level of $p < 0.05$ was obtained.

RESULTS

Fos INDUCTION IN RESPONSE TO VAGUS NERVE STIMULATION

In the control areas (the hypoglossal nucleus and the NAC core region), there were no significant differences in the expression of Fos positive cells between the VNS and sham groups [hypoglossal nucleus: $F_{(1,\ 11)} = 1.98$, $p > 0.1$; NAC core: $F_{(1,\ 11)} = 3.42$, $p = 0.09$]. **Figure 5** shows that electrical stimulation of the left vagus nerve significantly increased Fos-like immunoreactivity in each of the following brain regions bilaterally: NAC, CEA, BLA, HIP, LC, VLM, and NTS. Individual Two-Way ANOVAs using treatment (sham, 0.4mA) as the between subjects factor and hemisphere (left vs. right) as the within subjects factor indicated significant differences in Fos expression between the sham and VNS groups (NAC: $F_{(1,\ 11)} = 7.29$, $p < 0.05$; CEA: $F_{(1,\ 11)} = 9.36$, $p < 0.01$; BLA: $F_{(1,\ 11)} = 13.77$, $p < 0.01$; HIP: $F_{(1,\ 11)} = 21.88$, $p < 0.001$; LC: $F_{(1,\ 11)} = 12.2$, $p < 0.01$; VLM: $F_{(1,\ 11)} = 25.93$, $p < 0.001$; NTS: $F_{(1,\ 11)} = 10.79$, $p < 0.01$). *Post-hoc* tests comparing vagal stimulation and sham groups revealed that stimulation resulted in significantly more Fos expression within bilateral NAC (Left: $p < 0.05$; Right: $p < 0.05$), CEA (Left: $p < 0.05$; Right: $p < 0.05$), BLA (Left: $p < 0.01$; Right: $p < 0.01$), HIP (Left: $p < 0.05$; Right: $p < 0.001$), LC (Left: $p < 0.01$; Right: $p < 0.05$), VLM (Left: $p < 0.001$; Right: $p < 0.01$), and NTS (Left: $p < 0.05$; Right: $p < 0.01$) compared to sham stimulated animals.

STIMULATION SIDE PREDOMINANCE OF Fos EXPRESSION

Figure 5 also illustrates that electrical stimulation of the left vagus nerve not only caused bilateral activation in the above mentioned brain regions, but also induced a ipsilateral predominance of Fos expression in the LC and NTS. Within the LC, the Two-Way ANOVA revealed a hemisphere difference [$F_{(1,\ 11)} = 26.48$, $p < 0.001$] and interaction between treatment and hemisphere [$F_{(1,\ 11)} = 9.73$, $p < 0.01$]. *Post-hoc* tests comparing the two hemispheres detected a significant predominance of Fos expression in the left over right hemisphere of the vagus nerve stimulated animals ($p < 0.001$) but not of the sham controls. Within the NTS, the Two-Way ANOVA indicated a hemisphere difference [$F_{(1,\ 11)} = 8.47$, $p < 0.05$]. *Post-hoc* tests comparing the two hemispheres showed that both simulation and sham groups exhibited ipsilateral predominance of Fos expression (0.4 mA: $p < 0.05$; sham: $p < 0.05$). No other areas sampled exhibited side preferences in the expression of Fos.

DOUBLE LABELED (Fos+ /DBH+) CELLS IN LC, VLM, AND NTS

Figure 6 shows that left VNS activated noradrenergic activity in bilateral LC, bilateral VLM, and ipsilateral NTS. The photomicrographs are shown in **Figure 6**. Two-Way ANOVAs indicated that significant more double labeled (Fos+/DBH+) cells were induced by VNS in the LC [$F_{(1,\ 11)} = 10.09$, $p < 0.01$], VLM [$F_{(1,\ 11)} = 18.44$, $p < 0.01$], and NTS [$F_{(1,\ 11)} = 13.43$, $p < 0.01$]. *Post-hoc* tests revealed that VNS induced significant increase of the number of double labeled cells in the bilateral LC (Left: $p < 0.01$; Right: $p < 0.05$), bilateral VLM (Left: $p < 0.01$; Right: $p < 0.001$), and only left NTS (Left: $p < 0.01$) following left VNS.

Fos INDUCTION IN THE NTS IN RESPONSE TO EPINEPHRINE ADMINISTRATION

Injection of 0.1 or 0.5 mg/kg epinephrine induced strong Fos expression along five coronal levels of the NTS. A Two-Way ANOVA, with treatment (saline, 0.1 mg/kg, 0.5 mg/kg) as the between subjects factor and five NTS coronal levels as the within subjects factor showed that systemic epinephrine injection caused significant increases in the expression of Fos-like immunoreactivity within the NTS [$F_{(2,\ 15)} = 9.34$, $p < 0.01$]. *Post-hoc* tests found that both doses of epinephrine increased Fos expression significantly across all five levels of the NTS (all p-values < 0.05). This confirmed the effects of systemic injection of epinephrine in this study. In the control areas (the hypoglossal nucleus and the NAC core region), there were no differences in the expression of Fos positive cells between the epinephrine injected and saline groups [hypoglossal nucleus: $F_{(2,\ 15)} = 1.26$, $p > 0.1$; NAC core: $F_{(2,\ 15)} = 1.48$, $p > 0.1$].

Fos INDUCTION IN MEMORY ASSOCIATED AREAS FOLLOWING EPINEPHRINE ADMINISTRATION

Figure 7 shows that systemic administration of epinephrine induced significantly more Fos-like immunoreactivity in the following brain regions: NAC, CEA, BLA, HIP, LC, VLM, AP, and NTS. Because injection was given systemically and no hemisphere difference was found, Fos counts from both hemispheres were collapsed. Individual ANOVAs indicated significantly higher Fos expression in response to epinephrine injection (NAC [$F_{(2,\ 15)} = 7.12$, $p < 0.01$], CEA [$F_{(2,\ 15)} = 10.73$, $p < 0.01$], BLA [$F_{(2,\ 15)} = 16.69$, $p < 0.001$], HIP [$F_{(2,\ 15)} = 5.19$, $p < 0.05$], LC [$F_{(2,\ 15)} = 7.25$, $p < 0.01$], VLM [$F_{(2,\ 15)} = 10.92$, $p < 0.01$], and NTS [$F_{(2,\ 15)} = 9.34$, $p < 0.01$]. *Post-hoc* tests further indicated that (1) within the NAC, both doses of epinephrine caused more Fos-like immunoreactivity than in the saline group with no significant differences between the two doses (0.1 vs. saline, $p < 0.05$; 0.5 vs. saline, $p < 0.01$), (2) within CEA and

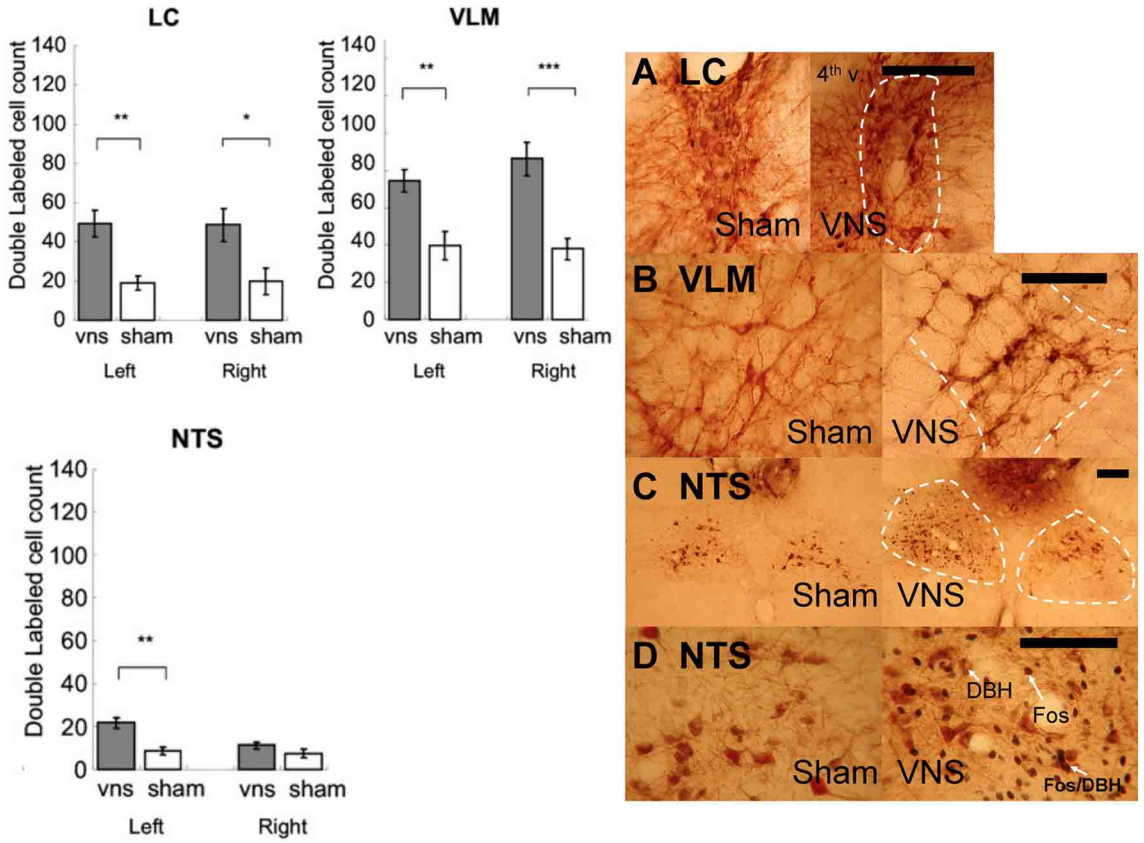


FIGURE 6 | Left vagus nerve stimulation activated noradrenergic cells in bilateral LC, bilateral VLM, and ipsilateral NTS. (*, **, and *** denotes significant difference between the compared groups with a *p*-value less than 0.05, 0.01, and 0.001, respectively). Photomicrographs of Fos/DBH labeling in the LC (A), VLM (B), NTS (C,D) following sham or vagus nerve stimulation. Scale bar = 200 um. (4^{th} v.: the fourth ventricle).

BLA, both doses of epinephrine induced more Fos expression than saline. Additionally, 0.5 mg/kg caused more Fos expression than 0.1 mg/kg (CEA: 0.5 mg/kg vs. 0.1 mg/kg, $p < 0.05$; 0.1 mg/kg vs. saline, $p < 0.05$; 0.5 mg/kg vs. saline, $p < 0.001$; BLA: 0.5 mg/kg vs. 0.1 mg/kg, $p < 0.01$; 0.1 mg/kg vs. saline, $p < 0.05$; 0.5 mg/kg vs. saline, $p < 0.001$), (3) within HIP, only 0.5 mg/kg induced more Fos-like immunoreactivity compared to saline (0.5 mg/kg vs. saline, $p < 0.01$), (4) within the LC, VLM, and NTS, both doses induced more Fos-like immunoreactivity compared with the saline group, with no significant difference between the two doses (LC: 0.5 mg/kg vs. saline, $p < 0.01$; 0.1 mg/kg vs. saline, $p < 0.05$; VLM: 0.5 mg/kg vs. saline, $p < 0.01$; 0.1 mg/kg vs. saline, $p < 0.01$; NTS: 0.5 mg/kg vs. saline, $p < 0.01$; 0.1 mg/kg vs. saline, $p < 0.01$).

DOUBLE LABELED (Fos+ /DBH+) CELLS IN LC, VLM, AND NTS

Figure 8 shows that systemic administration of epinephrine activated noradrenergic cells in the LC, VLM, and NTS. One-Way ANOVAs indicated that epinephrine injection significantly increased the number of double labeled cells in the LC [$F_{(2, 15)} = 5.28$, $p < 0.05$], VLM [$F_{(2, 15)} = 12.29$, $p < 0.001$], and NTS [$F_{(2, 15)} = 33.59$, $p < 0.0001$]. *Post-hoc* tests showed that both doses of epinephrine induced more double labeled cells in the LC (0.1 mg/kg vs. saline, $p < 0.05$; 0.5 mg/kg vs. saline, $p < 0.05$) and VLM (0.1 mg/kg vs. saline, $p < 0.001$; 0.5 mg/kg vs. saline, $p < 0.01$). However, in the NTS, although both doses of epinephrine induced significantly more double labeled cells than saline (0.1 mg/kg vs. saline, $p < 0.0001$; 0.5 mg/kg vs. saline, $p < 0.0001$), the 0.1 mg/kg dose induced significantly more double labeled cells than 0.5 mg/kg (0.5 mg/kg vs. 0.1 mg/kg, $p < 0.05$).

DOUBLE LABELED CELL PERCENTAGE OF TOTAL Fos+ LABELED CELLS IN LC, VLM, AND NTS

Figure 9A illustrates that VNS induces noradrenergic activation that accounts for 44% of the neuronal activity in the LC, 56% in the VLM, and 8.2% in the NTS. In addition, 0.1 mg/kg epinephrine injection induces noradrenergic activation that accounts for 25% of the neuronal activity in the LC, 39% in the VLM, and 9.6% in the NTS. Moreover, 24% of the Fos activation in the LC, 33% in the VLM, and 7.1% in the NTS are attributed to activated noradrenergic cells induced by 0.5 mg/kg epinephrine.

PERCENTAGE DISTRIBUTION OF DOUBLE LABELED CELLS IN THE LC, VLM, AND NTS

Figure 9B illustrates that, in the VNS group, out of the total double labeled cells of the three noradrenergic nuclei, 34% was contributed by the LC, 55% by the VLM, and 11% by the NTS.

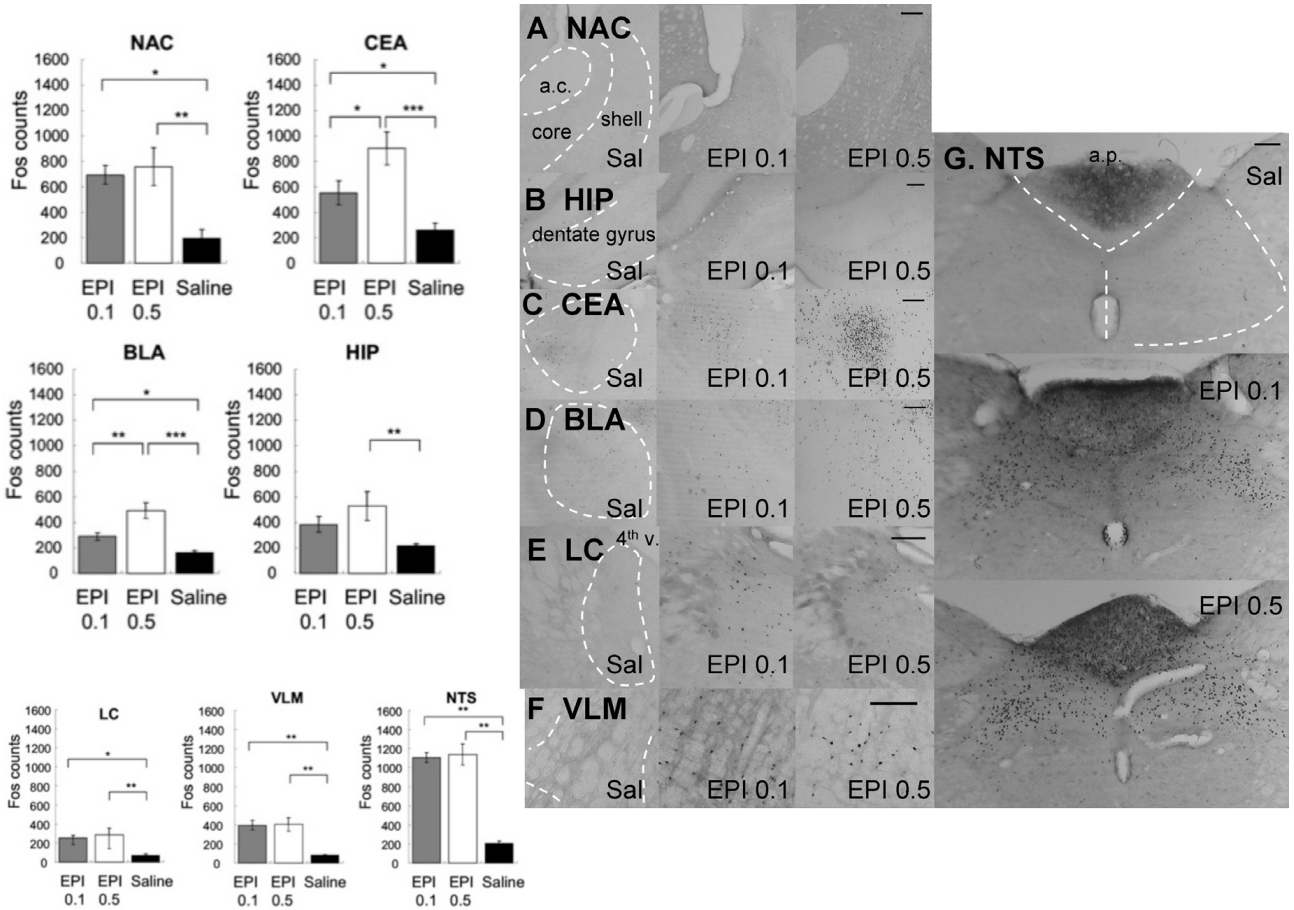


FIGURE 7 | Systemic injection of epinephrine induced significantly more Fos-like immunoreactivity in the NAC, CEA, BLA, HIP, LC, VLM, AP, and NTS. Because injection was given systemically and no hemisphere difference was found, Fos counts from both hemisphere were collapsed. (*, **, and *** denotes significant difference between the compared groups with a *p*-value less than 0.05, 0.01, and 0.001, respectively). Photomicrographs of Fos labeling in the NAC (A), HIP (B), CEA (C), BLA (D), LC (E), VLM (F), and NTS (G) following saline, 0.1 mg/kg or 0.5 mg/kg epinephrine injection. Scale bar = 200 um (a.c.: anterior commisure; shell: the shell region of the nucleus accumbens; core: the core region of the nucleus accumbens; 4th v.: the fourth ventricle; a.p.: area postrema).

In the epinephrine 0.1 mg/kg group, 20% was contributed by the LC, 47% by the VLM, and 33% by the NTS. In the epinephrine 0.5 mg/kg group, 24% was contributed by the LC, 47% by the VLM, and 29% by the NTS. The numerical summary of the immunostaining results from the two treatment groups is given as in **Figure 9C**.

DISCUSSION

Findings from this series of studies reveal an integral relationship between the arousal related hormone epinephrine and ascending fibers of the vagus nerve in influencing memory processing through interactions with central noradrenergic systems. The results from Experiment 1 demonstrated that increasing neural transmission along ascending vagal fibers following learning produces a significant and long lasting improvement in memory for emotional experiences. In the present experiment, altering the contingencies between expected rewards and experienced observations induced a level of emotional arousal in the Sham or VNS *shifted* groups that was reflected in increased latencies to traverse the length of the maze to receive only 1 as opposed to the expected 10 food pellets on the day of the *Shift*. A reduction in the expected level of reinforcement produces heightened levels of emotional frustration and changes in neuroendocrine functioning that influence the encoding of memory for these events (Crespi, 1942; Levine et al., 1972; Goldman et al., 1973). The vagus nerve plays an important role in this process by conveying to the brain, increased peripheral endocrine and autonomic activity that transpired as a result of the emotionally frustrating experience. Consequently, vagal nerve stimulation may produce beneficial effects on retention performance because increased vagal output to the brain may represent a natural consequence of elevated peripheral activity resulting from exposure to environmental events that are salient enough to induce adrenal secretion of epinephrine.

This view is supported by the finding that retention performance, as reflected by latencies to approach the food cup and consume the single food pellet reward 7 days following the *Shift* in reward magnitude, remained significantly longer than

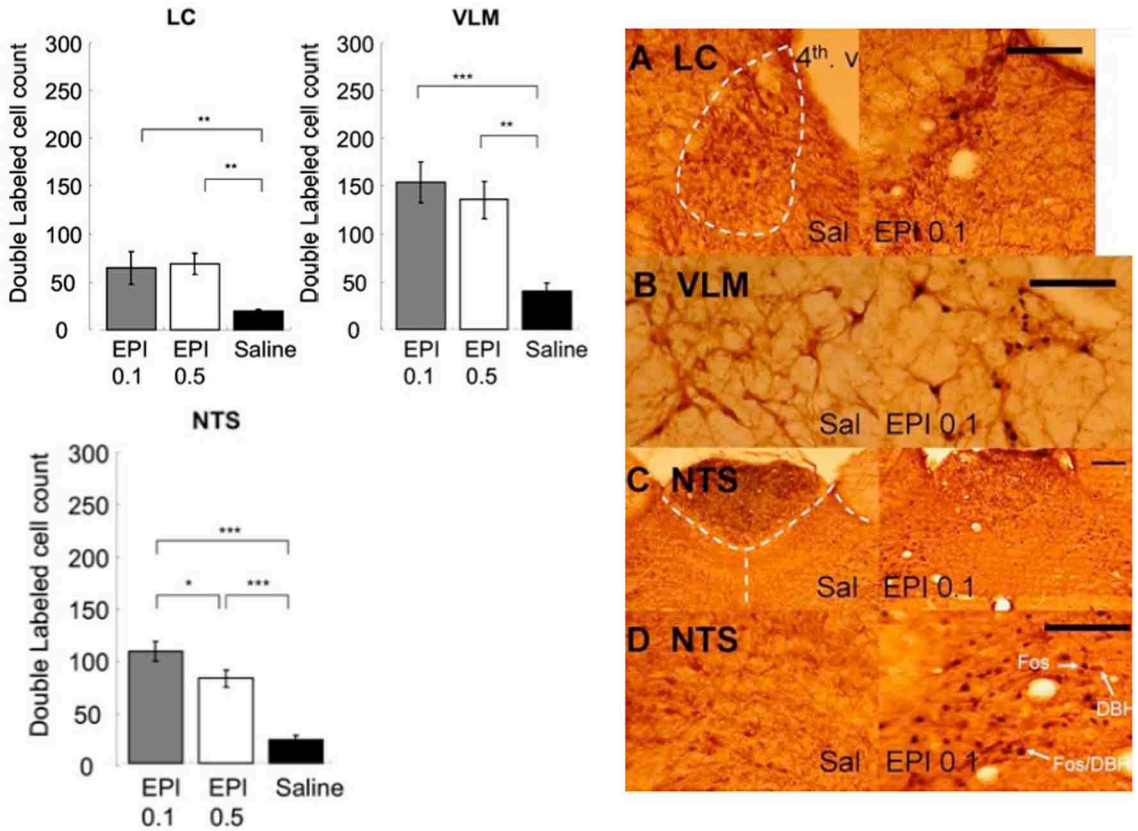
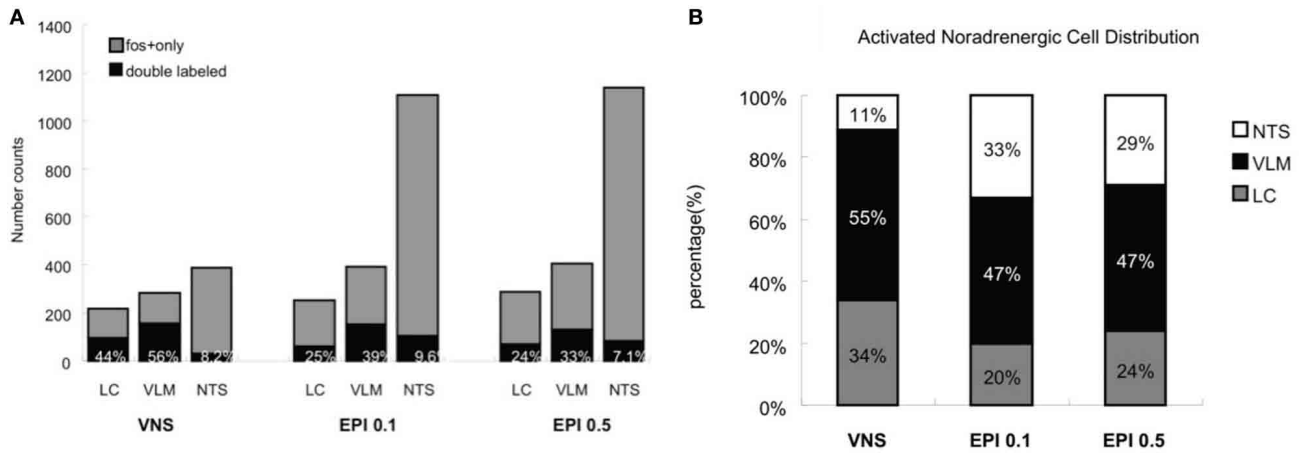


FIGURE 8 | Systemic injection of epinephrine activated noradrenergic cells in the LC, VLM, and NTS. (*, **, and *** denotes significant difference between the compared groups with a *p*-value less than 0.05, 0.01, and 0.001, respectively). Photomicrographs of Fos/DBH labeling in the LC (A), VLM (B), NTS (C,D) following saline or epinephrine injection (0.1 mg/kg). Scale bar = 200 um. (4^{th} v.: the fourth ventricle).

the *No-Shift* groups. In contrast, the latencies of the Sham-stimulated groups that also experienced the *Shift* and reduced food rewards were no different than those of *No-Shift* controls on this delayed retention test. Moreover, the latencies of the VNS group were also significantly longer on the 7 day delayed test than the *Shifted* Sham-stimulated group. These as well as other findings demonstrate that similar to epinephrine injections, VNS produces bimodal effects on retention performance. For example, whereas only moderate doses of epinephrine improve memory, higher doses of this hormone such as those that result from traumatic experiences that are not well retained in humans produce amnesia (Gold et al., 1977; Izquierdo and McGaugh, 1987). Similarly, the intensity of current applied to the vagus nerve in the current study (0.4 mA) has been shown to improve memory in one-trial avoidance tasks in rodents and declarative memory in humans (i.e., 0.5 mA) whereas higher levels of stimulation are known to produce memory impairments (Clark et al., 1998). Thus, the consequences on mnemonic processing in response to graded levels of vagal input to the brain mirror the dose-dependent inverted-U actions of peripheral hormones such as epinephrine on memory formation. Consistent with this view, our findings indicate that up-regulation of vagal neural activity improves memory formation as evidenced by the enhanced retention performance observed in the vagal stimulated groups up to seven days after learning.

Increasing impulse flow from the vagus nerve to the brain is an effective method of influencing brain processes that not only encode memory for experiences that are emotionally arousing but this treatment has also been shown to improve both recognition and recall performance in humans trained on serial word lists tasks (Clark et al., 1999; Ghacibeh et al., 2006), increase non-associative learning in animals (Wang et al., 2005) and to facilitate attention and alertness in children implanted with vagal electrodes (Kossoff and Pyzik, 2004). The findings from the current study corroborate previous reports and extend these results by revealing that the capacity for vagal input to influence mnemonic processing is not limited to learning conditions involving noxious stimuli but activity along this nerve is an essential component of the processes involved in modulating a broad range of new emotional learning experiences into long term memory.

The results from Experiment 2 demonstrated that epinephrine increases firing discharge along afferent fibers of the vagus nerve and this change persisted for up to one hour post-injection. The epinephrine-induced increase in vagal firing was accompanied by significant elevations in extracellular concentrations of



C

Fos positive cell counts following EPI or VNS	NAC	CEA	BLA	HIP	LC	VLM	NTS
VNS	584 ± 117	436 ± 48	577 ± 79	1001±119	218 ± 32	283 ± 24	388 ± 58
EPI 0.1	694 ± 71	553 ± 94	288 ± 27	385 ± 63	252 ± 29	394 ± 51	1107 ± 125
EPI 0.5	758 ± 149	902 ± 130	492 ± 62	527 ±111	287 ± 65	404 ± 72	1138 ± 264

Double labeled cell counts following EPI or VNS	LC	VLM	NTS
VNS	97±14	160±14	32±3
EPI 0.1	65±17	154±21	107±9
EPI 0.5	69±11	135±19	81±8

FIGURE 9 | (A) Percentage of noradrenergic of cell activation. Vagus nerve stimulation induces noradrenergic activation that accounts for 44% of the neuronal activity in the LC, 56% in the VLM, and 8.2% in the NTS. In addition, 0.1 mg/kg epinephrine injection induces noradrenergic activation that accounts for 25% of the neuronal activity in the LC, 39% in the VLM, and 9.6% in the NTS. Moreover, 24% of the Fos activation in the LC, 33% in the VLM, and 7.1% in the NTS are attributed to activated noradrenergic cells induced by 0.5 mg/kg epinephrine. **(B)** Activated noradrenergic cell distribution. In the vagus nerve stimulation group, out of the total double labeled cells of the three noradrenergic nuclei, 34% was contributed by the LC, 55% by the VLM, and 11% by the NTS. In the epinephrine 0.1 mg/kg group, 20% was contributed by the LC, 47% by the VLM, and 33% by the NTS. In the epinephrine 0.5 mg/kg group, 24% was contributed by the LC, 47% by the VLM, and 29% by the NTS. **(C)** A numerical summary of the different activation patterns induced by vagus nerve stimulation or systemic epinephrine injection. The findings indicate that heightened levels of plasma epinephrine and increased vagal activity are capable of affecting in a similar fashion, the cellular activity of noradrenergic neurons and its output areas. Epinephrine injection activates high levels of NTS noradrenergic neurons whereas the vagus nerve stimulation recruits more LC noradrenergic activity. As a consequence, epinephrine injected animals exhibited more cell activation in NTS noradrenergic output areas (CEA, NAC) and vagus nerve stimulation animals showed more cell activation in LC noradrenergic output areas (HIP, BLA).

norepinephrine in the basolateral amygdala that remained high for up to 120 min post-injection. Additionally, the results suggest that the excitatory actions of epinephrine on vagal nerve discharge are mediated through influences on β-adrenergic receptors. The β-adrenergic blocker sotalol (2 mg/kg) given with epinephrine was effective in preventing epinephrine related changes in vagal activity and also blocked the capacity for this hormone to produce in appreciable changes in amygdala norepinephrine release. However, peripheral adrenergic blockade with sotalol was delayed to 60 min post-epinephrine injection, only the higher dose (4 mg/kg in the EPI + SOT4 group) was effective in reversing the effects of epinephrine on vagal nerve discharge. But the counteracting effects were not present immediately. That is, epinephrine-induced vagal activity and amygdala norepinephrine levels were not reduced to control levels until 40 and 120 min, respectively post-sotalol injection. Furthermore, in groups where no increases in vagal discharge were observed, no increases in norepinephrine release were detected in the basolateral amygdala. For convenience, these groups (SAL, SAL + SOT4, SOT2 + SAL, SOT2 + EPI) were referred to as the control groups. Collectively, our results indicate that changes in vagal discharge and amygdala norepinephrine release are mediated in part by β-adrenergic receptors in response to the arousal related hormone epinephrine. Once adrenergic activity is enhanced by epinephrine, it requires a higher dose of an adrenergic antagonist to counteract epinephrine effects.

EPINEPHRINE vs. SALINE

Findings from the current study show that a single injection of the hormone at a dose of 0.3 mg/kg significantly increased neural impulses propagated along ascending vagal fibers for approximately 50 min. The magnitude of the increase peaked at 30% above baseline 30 min post-epinephrine injection. The concomitant microdialysis data show that peripheral epinephrine elevates noradrenergic output in the basolateral amygdala in a pattern that is similar to the changes this hormone induces initially on the firing rates of vagal fibers. Unlike vagal activity however, amygdala norepinephrine release induced by epinephrine remained elevated for the entire collection period (i.e., 180 min). Norepinephrine levels were 44% higher than baseline at 40 min post injection, 77% higher at 180 min post injection indicating an increasing elevation of central noradrenergic activity in response to peripheral epinephrine. Consequently, we hypothesize that the role of ascending vagal fibers may play an integral role in initiating, as opposed to maintaining brain noradrenergic activation in response to increased peripheral adrenergic activity. Therefore, central noradrenergic activation, induced by systemic epinephrine injection may be initiated by increased neural impulses carried by ascending vagal fibers and then maintained by other mechanisms.

Neural activity propagated along vagal afferent fibers may recruit additional mechanisms that affect long-term changes in CNS functioning. In fact, cAMP has been reported to accumulate in the vagus nerve following incubation in a medium containing epinephrine (Roch and Salamin, 1977). The change in vagal concentrations of cAMP has been reported to increase as much as 10–50-fold following incubation in solutions containing the β adrenergic agonist isopreteronol (Schreurs et al., 1986). Given the importance of cAMP as an important second messenger essential for ligand gated neurotransmission, the accumulated cAMP molecules might be trafficked to areas that undergo cAMP-PKA-dependent transmission, such as the central nervous system. It is known that vagal afferents influence the level of cAMP in central sites of termination such as the NTS, which then determine the state of activation in post-synaptic NTS neurons (Browning and Travagli, 2006). Noradrenergic cells are one major type of post-synaptic NTS neurons. Given the fact that noradrenergic neurotransmission is mostly cAMP PKA-dependent, accumulated cAMP in the vagus nerve following epinephrine treatment might be utilized to promote noradrenergic activity in the NTS. In fact, in severely stressful conditions that are known to enhance peripheral adrenergic activity, NTS neurons are up-regulated with PKA, Fos, and tyrosine hydroxylase immunoreactivity (Benavides et al., 2005). This is consistent with the finding that noradrenergic neurons are activated in response to increased vagal activity or peripheral adrenergic activity. With the activation of NTS noradrenergic neurons, norepinephrine release in the basolateral amygdala can then be initiated and maintained.

Findings emerging from the final study reveal that systemic epinephrine injection and VNS induce similar patterns of neuronal activation within brain regions associated with memory. Our findings indicate that treatments that raise circulating levels of epinephrine in the periphery or increase discharge along ascending fibers of the vagus nerve produce similar changes in the expression of Fos proteins in noradrenergic neurons in the NTS, LC, VLM, and their areas of termination. The results show that central noradrenergic nuclei represent a primary target of peripheral systems that are engaged by exposure to emotionally arousing events. In turn, activation of noradrenergic release in each of the limbic circuits identified in this study constitutes areas wherein the beneficial effects of heightened levels of arousal in the periphery influence the effective storage of events into memory.

When comparing the number of Fos expressing cells observed in response to either vagal stimulation or epinephrine injections, the two doses of epinephrine recruited nearly three times as many Fos neurons as that produced by vagal stimulation (EPI 0.1 mg/kg: 1107 ± 125; EPI 0.5 mg/kg: 1128 ± 264; VNS: 388.58 ± 58). The difference in treatments most likely accounts for this observation. For example, in contrast to the unilateral stimulation of the vagus nerve, both vagal nerves are most likely activated in response to the systemic epinephrine injections and the observed effects may reflect the central consequences of increasing discharge along both vagi.

Even though NTS neurons were activated to a different degree by the separate treatments, the patterns of increased activation within other more rostral structures were uniformly bilateral. Systemic injection of epinephrine produced equivalent activation in both hemispheres within all of the examined areas, i.e., NTS, VLM, LC, BLA, CEA, HIP, and NAC. Similar findings were observed after vagal stimulation, with the exception of a clear ipsilateral predominance of Fos expression in the NTS and LC. Consistent with previous studies (Gieroba and Blessing, 1994; Osharina et al., 2006), this observation suggests that the propagation of ascending information from the vagus bifurcates at the level of the NTS and LC. Vagal afferents first decussate at the spinal trigeminal tract before entering the NTS (Kalia and Mesulam, 1980; Ranson et al., 1993; Norgren and Smith, 1994). NTS neurons then project to a wide range of medullary and pontine areas bilaterally (Otake et al., 1992). Based upon these observations, VLM projecting neurons in the NTS send out nearly equivalent innervations to both hemispheres whereas LC projecting neurons show an ipsilateral predominance. LC neurons subsequently project to several forebrain areas bilaterally with an ipsilateral predominance (Jones and Moore, 1977; Espana and Berridge, 2006).

Animal studies that investigated the effects of VNS on forebrain areas are relatively sparse. According to currently available evidence, noradrenergic output is up-regulated in the amygdala (Hassert et al., 2004), hippocampus, and cortex (Roosevelt et al., 2006). Additionally, the Roosevelt et al. study (2006) reported a bilateral activation pattern following VNS at an intensity of 0.5 mA in the hippocampus, or of 1.0 mA in both the hippocampus and cortex. These findings are consistent with the Fos activation patterns obtained in our study. That is, we observed equivalent increases in both hemispheres following VNS at an intensity of 0.4 mA in noradrenergic output areas, including NAC, CEA, BLA, and HIP.

The findings from neuroimaging studies that investigated the clinical therapeutic effects of VNS in treating epilepsy and depression are generally congruent with our findings.

Significant increases in cerebral blood flow after acute left vagal stimulation was noted bilaterally in the hippocampus, amygdala, orbitofrontal gyri, cingulate, thalami, hypothalami, and the anterior insula (Henry et al., 1998). However, changes observed after vagal stimulation in a fMRI study of patients with intractable depression involved increased blood oxygenation level in bilateral orbitofrontal and parieto-occipital cortex, left temporal cortex, amygdala, and the hypothalamus (Bohning et al., 2001). In patients with refractory epilepsy, VNS-induced activation was detected in bilateral thalamus, insular cortices, postcentral gyri and inferomedial occipital gyri, left basal ganglia, and right posterior superior temporal gyrus (Narayanan et al., 2002). Although side predominance was observed in some of the neuroimaging studies, neuroimaging evidence is still in agreement with our findings that VNS induces equivalently bilateral activation in brain areas that are secondary to the stimulation site, i.e., limbic areas in our study.

NORADRENERGIC ACTIVATION PATTERNS

Although the activation patterns (indicated by Fos data) within the lower brain regions appear similar following the two treatments, double labeling data found that each of the noradrenergic nuclei may have different roles in enhancing central noradrenergic activity. That is, VNS induces a higher percentage of noradrenergic activation in the LC (44%) and VLM (56%) than epinephrine injection whereas epinephrine injection recruits a higher percentage (9.6% by 0.1 mg/kg) of noradrenergic activation in the NTS. The contribution of each nucleus is best shown by the distribution of activated noradrenergic (double labeled) neurons within the three noradrenergic regions. Results indicate that the LC noradrenergic neurons are more involved in the VNS activation whereas the NTS is more important in the epinephrine injection activation. VLM A1 cells, however, account for nearly half of the noradrenergic activation induced by either treatment, with a higher predominance following VNS.

In conclusion, the collective findings demonstrate an integral role of epinephrine and the vagus nerve in influencing central noradrenergic systems ascribed a role in processing memory for emotionally arousing experiences. The findings also illustrate how interactions between these two peripheral endocrine and autonomic systems serve an important function in elevating and maintaining heightened levels of central activity in response to emotionally salient events. This comprehensive analysis of vagal nerve functioning in memory, in regulating norepinephrine output in the amygdala and in influence activity in norepinephrine containing cell bodies in the CNS reveals the important contribution of this nerve in facilitating "*peripheral to central*" communication during critical states of emotional arousal.

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