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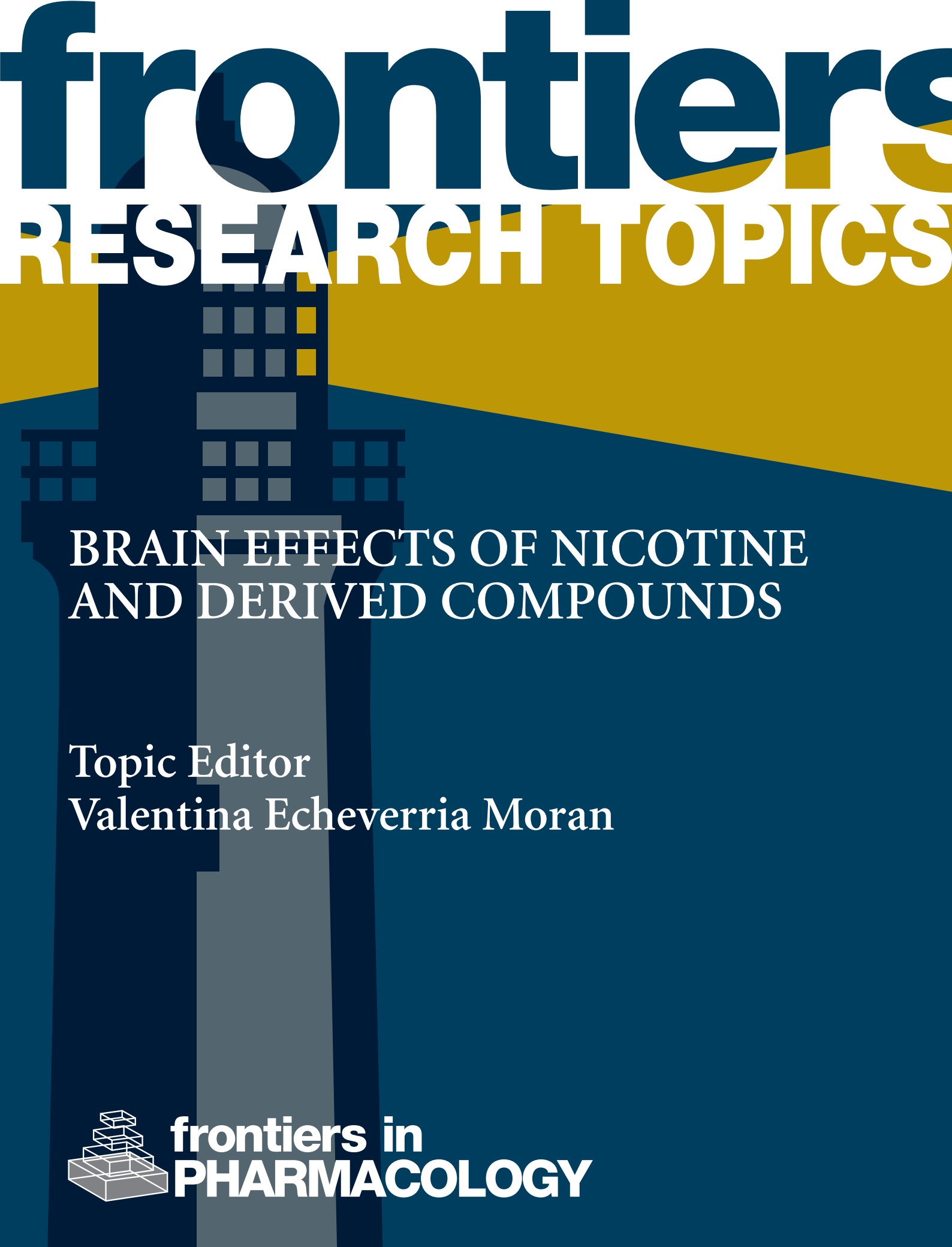

BRAIN EFFECTS OF NICOTINE AND DERIVED COMPOUNDS

Topic Editor
Valentina Echeverria Moran

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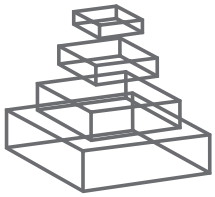

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BRAIN EFFECTS OF NICOTINE AND DERIVED COMPOUNDS

Topic Editor:
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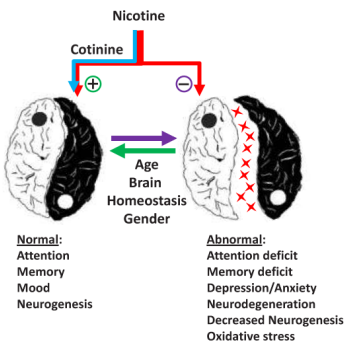

This figure is the result of the creative work of Dr. Alexandre Iarkov

Epidemiological studies have associated tobacco consumption with a lower incidence of Alzheimer's disease (AD) and Parkinson's disease (PD). The neuroprotective effect of tobacco has been mainly attributed to the stimulation by nicotine of the a7 nicotinic acetylcholine receptors (nAChRs), which are implicated in neuronal survival, attention, and memory. A reduction in cholinergic function including lower levels of the expression of nAChRs in the hippocampus correlates with memory impairment in AD and schizophrenia. nicotine main metabolite cotinine shows similar neuroprotective and mnemonic properties. Also cotinine reduced Alzheimer's pathology in a mouse model of the disease. Tobacco-derived compounds can be beneficial in a broad range of neurological disorders such as schizophrenia, AD, attention-deficit hyperactivity disorder, and PD as well as in diminishing tobacco withdrawal. In the latest years numerous new discoveries in this research area have been achieved and a Research Topic publication is needed.

Table of Contents

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EDITORIAL
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Brain effects of nicotine and derived compounds

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Recent evidence in the field of tobacco research has provided new insights about the mechanisms underlying the effects of nicotine and its derivatives (NAD) in human and animal behavior and pathology. This topic covers several areas of clinical and basic research focused on the effect of NAD on brain function and behavior during development and adulthood. As expressed by Spijker and colleagues (Counotte et al., 2012), there is a Ying and a Yang in the effect of nicotine and its metabolites on the brain (**Figure 1**).

The influence of NAD on addictive behavior and the mechanisms involved are of great interest for public health. One of our articles explores the molecular mechanisms associated with the differences in addiction to tobacco between genders. They investigated the effect of nicotine, cotinine, and anabaseine on the estrogen biosynthesis in the brain. They show experimental evidence that these compounds affect estrogen synthesis in the brain via the modulation of the last enzyme of the estrogen biosynthesis named aromatase. Based on this evidence they discussed the hypothesis that brain aromatase mediates the effect of nicotine in the brain resulting in sex differences in smoking behavior. Prenatal NAD exposure may affect addictive behavior later in life, several studies have shown that maternal smoking and prenatal nicotine affect brain development and motivated behavior. In here, Harrod et al. show evidence that prenatal nicotine exposure have reinforcing influence over the addictive properties of methamphetamine, affecting vulnerability to addictive behavior in the adulthood (Harrod et al., 2012). Similarly Chen et al. discuss the influence of prenatal nicotine from tobacco smoking on feeding behavior in adulthood as well as the potential of nicotine as a weight loss treatment (Chen et al., 2012). Furthermore, other addictive behaviors such as alcohol dependence may heavily influence the effect of NAD on cognitive abilities. For example, Durazzo et al. found increased neurocognitive deficits in alcohol dependent individuals that also were smokers when compared to non-smokers (Durazzo et al., 2012).

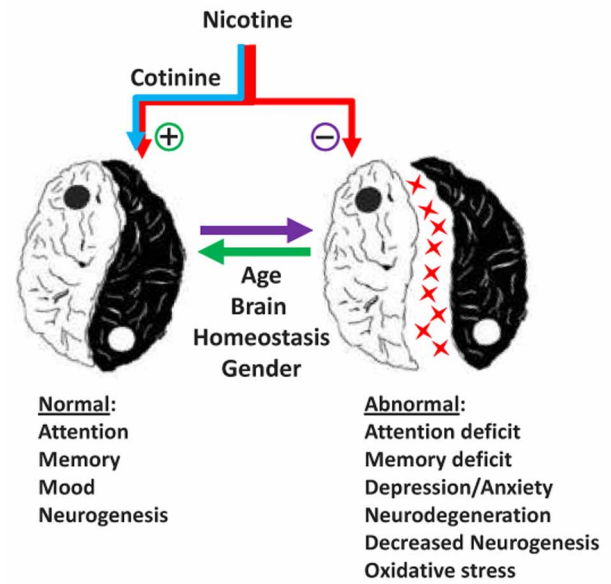

FIGURE 1 | The Ying and Yang of the effects Nicotine and Cotinine in the adult brain.

On the other hand, the effect of nicotine on neuroplasticity is controversial, and its improvement and deterioration by NAD have been described. In fact, the investigation by several research groups about the effect of nicotine on attention (Kadir et al., 2006) and learning and memory have given heterogeneous results (Smith et al., 2006). Grundey et al. (2012) show new evidence of a negative effect of nicotine spray on facilitatory plasticity and a diminished reduction in excitability after transcranial direct current stimulation. These results differ from the effects observed after chronic nicotine administration. They attributed these differences to the adaptive nicotinic receptor changes induced by continue nicotine exposure. These heterogeneous effects can be the result of a differential effect of NADs according to the brain state. Counotte et al. (2012) discuss the diverse responses to nicotine on attention, depending on several factors including the extent of exposure (acute vs. chronic), smoking behavior, the developmental stage at which the brain is exposed to nicotine and the presence of psychiatric conditions such as, schizophrenia and Alzheimer's Disease (AD). Psychiatric conditions and/or chronic nicotine exposure may also alter the expression or responsiveness of the cholinergic receptors in the brain and consequently the effect of nicotine on higher order cognitive functions.

Some epidemiological studies have shown data suggesting an inverse relationship between tobacco consumption and the development of AD (Lee, 1994). It has also been found post-mortem that the levels of b-amyloid peptides (Aβ) (considered the neurotoxic agents in AD brains) were significantly decreased in the brains of smoking AD patients compared to non-smokers with the disease. The putative beneficial effect of tobacco has been

mainly attributed to nicotine, which has been reported to improve cognitive abilities and reduce plaques in a mouse model of AD (Nordberg et al., 2002). However, nicotine has not demonstrated in clinical studies to be a useful treatment for AD (Lopez-Arrieta et al., 2001). Since nicotinic acetylcholine receptors (nAChRs) play an important role in attention and learning and memory, the positive effects of nicotine on memory have been mostly credited to the activation of these receptors (Sabbagh et al., 2002). A change in the function of nAChRs will influence the release and activity of other neurotransmitters whose release is controlled by these receptors including glutamate, dopamine, serotonin, glycine, and g aminobutyric acid (GABA) (Livingstone and Wonnacott, 2009).

Knott et al. show evidence implicating the N-methyl-d-aspartate receptor (NMDAR) in the beneficial effect of nicotine over auditory, sensory, memory and attention in a human ketamine model of schizophrenia (Knott et al., 2012). In addition, it is discussed the beneficial effect of cotinine, the main metabolite of nicotine, improving memory and attention in several psychiatric conditions including AD (Echeverria et al., 2011), post-traumatic stress disorder (Zeitlin et al., 2012), and schizophrenia (Buccafusco and Terry, 2009). This evidence permits to hypothesize that many of the beneficial effects of nicotine may be at least in part the result of cotinine's actions in the brain (Echeverria and Zeitlin, 2012). The involvement of nicotine action in AD is a complex scientific question, and still needs to be defined whether cognitive impairment in AD is mainly induced by a decrease in the number of nAChRs and/or their function induced by neurotoxic forms of Aβ. In this topic, Zappettini et al. show evidence suggesting that Aβ1-40 inhibits the release of glycine in the hippocampus throughout a mechanism involving the nAChRs but not the muscarinic receptors (Zappettini et al., 2012).

All together this topic gives an actualized view of the NAD effects in aspects of addictive behavior, attention, neuroplasticity, and learning and memory under physiological and pathological conditions.

ACKNOWLEDGMENTS

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Offspring of prenatal IV nicotine exposure exhibit increased sensitivity to the reinforcing effects of methamphetamine

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Maternal smoking during pregnancy is associated with increased substance abuse in offspring. Preclinical research shows that *in utero* exposure to nicotine, the primary psychoactive compound in tobacco smoke, influences the neurodevelopment of reward systems and alters motivated behavior in offspring. The present study determined if prenatal nicotine (PN) exposure altered the sensitivity to the reinforcing and aversive effects of methamphetamine (METH) in offspring using a low dose, intravenous (IV) exposure method. Pregnant dams were administered nicotine (0.05 mg/kg/injection) or prenatal saline (PS) 3×/day on gestational days 8–21, and adult offspring were tested using METH self-administration (experiment 1) or METH-induced conditioned taste aversion (CTA; experiment 2) procedures. For METH self-administration, animals were trained to respond for IV METH (0.05 mg/kg/infusion; fixed-ratio 3) and they were tested on varying doses of the reinforcer (0.0005–1.0 mg/kg/infusion). For METH CTA, rats received three saccharin and METH pairings (0, 0.3, or 0.5 mg/kg, sc) followed by 14 daily extinction trials. Experiment 1: PN and PS animals exhibited inverted U-shaped dose-response curves; however, the PN animal's curve was shifted to the left, suggesting PN animals were more sensitive to the reinforcing effects of METH. Experiment 2: METH CTA was acquired in a dose-dependent manner and the factor of PN exposure was not related to the acquisition or extinction of METH-induced CTA. There were no sex differences in either experiment. These results indicate that IV PN-exposed adult offspring exhibited increased sensitivity to IV METH. This suggests that PN exposure, via maternal smoking, will alter the reinforcing effects of METH during later stages of development, and furthermore, will influence substance use vulnerability in adult human offspring.

Keywords: maternal smoking, prenatal nicotine, intravenous, methamphetamine, self-administration, conditioned taste aversion

INTRODUCTION

Maternal smoking during pregnancy imparts multiple health risks on the fetus (Castles et al., 1999; Ernst et al., 2001; Winzer-Serhan, 2008; Cornelius and Day, 2009). Aside from the well documented effects of maternal smoking to produce low birth weight, restricted intrauterine growth, and increased incidence of sudden infant death syndrome, it is also apparent that offspring are at increased risk of developing substance use disorder. According to clinical research, tobacco smoke-exposed offspring exhibit an increased likelihood of drug abuse if they initiate drug taking behaviors during adolescence (Kandel et al., 1994; Weissman et al., 1999; Brennan et al., 2002; Buka et al., 2003).

Various animal models of prenatal nicotine (PN) exposure have been used to elucidate the influence of nicotine on the neurobehavioral development of offspring (Dwyer et al., 2009; Heath and Picciotto, 2009). In these models, PN is administered to rodents either continuously via osmotic minipump (Slotkin et al., 1987; Levin et al., 1996; Franke et al., 2007), orally, through drinking water (Zhu et al., 1996; Pauly et al., 2004; Paz et al., 2007; Schneider et al., 2010), or through intravenous (IV) injection (LeSage et al., 2006; Harrod et al., 2011; Lacy et al., 2011). Multiple experiments demonstrate that PN exposure alone produces alterations in the neurodevelopment of the mesocorticolimbic dopamine (DA) system, which in part mediates motivated behavior. Such PN-induced changes are hypothesized to mediate alterations in the behavioral repertoire of adolescent and adult offspring exposed to PN (Pauly et al., 2004; LeSage et al., 2006; Franke et al., 2008; Lacy et al., 2011). For example, experiments using the continuous route of PN exposure resulted in decreased striatal DA content in weanling rats (Richardson and Tizabi, 1994), increased *c-fos* expression in the infralimbic cortex and nucleus accumbens core (Park et al., 2006), and decreased nicotine-evoked DA release in the nucleus accumbens shell of adolescent rats (Kane et al., 2004). In drug self-administration experiments, adolescent offspring exposed to continuous PN acquired cocaine self-administration at a higher unit dose of drug relative to prenatal saline (PS) controls. This finding suggests that PN exposure altered the reinforcing effects of cocaine (Franke et al., 2008). Adult, PN-exposed female offspring

self-administered a greater number of nicotine infusions than PS rats following a period of forced abstinence, which suggests that PN-exposed offspring exhibited increased motivation to respond for nicotine (Levin et al., 2006). Together, these experiments indicate that continuous PN exposure alters the neurodevelopment of the mesocorticolimbic DA system and that such changes impact the motivation for drug reward.

The present experiments determined if IV PN exposure altered the motivational effects of the highly abused drug methamphetamine (METH) in adult offspring. It is of interest to use the IV route of administration to deliver nicotine because it closely models the nicotine pharmacokinetics of tobacco smoke inhalation (Russell and Feyerabend, 1978; Mactutus, 1989; Booze et al., 1999; Benowitz et al., 2009). The elimination half-life for nicotine 0.05 mg/kg/injection is approximately 50 min (Booze et al., 1999), and so this method represents a unique exposure model in that the dam and fetuses experience the bolus delivery of nicotine to the brain followed by a rapid and precipitous clearance (Russell and Feyerabend, 1978; Mactutus, 1989). Moreover, this method may be used to deliver less overall daily amounts of nicotine relative to other exposure models, while still providing a translationally relevant exposure (see Dwyer et al., 2008). IV PN exposure produced changes in brain-derived neurotrophic factor throughout the mesocorticolimbic DA system of adolescent offspring (Harrod et al., 2011). Pre-weanling offspring exhibited hypoactive locomotor activity in response to a novel context (LeSage et al., 2006), and showed deficits in sensorimotor gating of the acoustic startle response (Lacy et al., 2011). Furthermore, adult offspring showed increased motivation for sucrose reward, relative to controls, when rats responded according to a progressive-ratio schedule of reinforcement; and in this study there were no effects of prenatal treatment when fixed-ratio (FR) schedules of reinforcement were used (Lacy et al., 2012). These findings demonstrate that administration of PN via the IV route produces changes in the neurodevelopment of motivational brain systems, and that offspring exhibit behavioral deficits during various stages of neurodevelopment, relative to controls.

The present experiments tested the hypothesis that IV PN exposure will result in offspring that exhibit altered sensitivity to the rewarding and aversive effects of METH using two conditioning procedures: drug self-administration and conditioned taste aversion (CTA). Previous research shows that PN-exposed offspring exhibited altered responding for IV nicotine (Levin et al., 2006) and IV cocaine (Franke et al., 2008), according the drug self-administration procedure. To date, no experiments have determined the effects of PN on the rewarding or aversive effects of METH in offspring. Investigating METH is of interest because amphetamines alter synaptic monoamine levels in the mesocorticolimbic system differently than other abused drugs, such as nicotine and cocaine. Amphetamines redistribute DA and other monoamines from the presynaptic terminal to the synapse by producing neurotransmitter release from vesicular monoamine transporters; by inhibiting monoamine oxidase, and by reversing DA transporter function (Sulzer et al., 2005). Cocaine and nicotine increase synaptic DA levels by blockade of the DA transporter and nicotinic receptor stimulated DA release, respectively (Laviolette and van der Kooy, 2004; Gether et al., 2006).

Two separate experiments were therefore conducted to determine if PN offspring exhibit altered appetitive or aversive conditioning to METH during adulthood. In experiment 1, PN animals prenatally exposed to IV nicotine were trained to self-administer METH (0.05 mg/kg/infusion) according to a FR schedule of reinforcement. Animals were then tested on multiple doses of IV METH, ranging from 0.0005 to 1.0 mg/kg/infusion, to assess adult offspring's sensitivity to the reinforcing effects of the drug. Experiment 2 determined if IV PN exposure alters acquisition and/or extinction of METH-induced CTA, the standard method used to assess the aversive effects of an unconditioned stimulus (US). Potential differences in the sensitivity to the aversive effects of METH were of interest because the trajectory for the escalation of drug use may be determined, in part, by the relative response to the aversive and rewarding effects of a drug (Davis and Riley, 2010). This is the first experiment to assess drug-induced CTA in offspring of PN exposure. Adult offspring consumed a saccharin conditional stimulus (CS), which was paired with the METH US (0, 0.3, or 0.56 mg/kg; sc) to produce CTA learning, and extinction of the conditioned response (CR) was measured, as well.

MATERIALS AND METHODS

ANIMALS

A total of 80 female and 30 male adult, nulliparous Sprague-Dawley rats were acquired from Harlan Industries, Inc., (Indianapolis, IN, USA). All rats were transported to the animal care facilities in the psychology department at the University of South Carolina and rodent food (ProLab Rat/Mouse/Hamster Chow 3000) and water were provided *ad libitum* throughout the course of the experiments, except when otherwise specified. All animal cages were provided with Nylabones (Nylabone, Inc.; long lasting durable chew-original; Neptune, NJ, USA) and Nestlets (NestletsT; Ancare, Bellmore, NY, USA), for purposes of environmental enrichment throughout the duration of the study. A Nylabone was replaced if it was thoroughly chewed, and one Nestlet nesting product was placed in the animals' cage when the cage was changed, which occurred 2×/week. The animal colony was maintained at $21 \pm 2^{\circ}$C, $50 \pm 10\%$ relative humidity, and a 12L:12D cycle with lights on at 07:00 hours. The protocol for this research methodology was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of South Carolina.

BREEDING

Following a 7-day habituation period, female rats were housed three per cage, and one male was placed with each triad from approximately 17:00–09:00. Vaginal lavage samples were analyzed daily with a microscope to determine if a sample was sperm-positive. When a sample was identified as sperm-positive, the corresponding female was single-caged and that day was considered gestational day (GD) 0. The weights of the pregnant dams were recorded daily during pregnancy.

SURGERY: INDWELLING, INTERIORIZED JUGULAR CATHETERS

The internalized jugular catheters used in the present experiment are commercially available from Harlan Industries. The catheterization was performed at Harlan Industries (Indianapolis, IN,

USA) according the methods of Mactutus et al. (1994) prior to breeding. Briefly, animals were anesthetized with a mixture of ketamine hydrochloride (100 mg/kg/ml) and xylazine (3.3 mg/kg/ml). Following anesthesia a sterile Intracath IV catheter (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) with a Luer-Lok injection cap (Medex, Inc., Carlsbad, CA, USA) was implanted dorsally in a subcutaneous pouch. The distal end of the catheter was inserted into the left jugular vein, advanced toward the heart, and the catheter was bound with sterile suture. Animals were kept under periodic post-operative observation and returned to the colony upon recovery. On the day following surgery, catheters were flushed with 0.2 ml of heparinized saline.

PN AND PS ADMINISTRATION

Pregnant dams were randomly assigned to either the PN (0.05 mg/kg/ml) or PS groups. Nicotine or saline was administered 3×/day via internalized IV catheters from GD 8–21. An organism's susceptibility to a teratogen can vary depending on the stage of development (Vorhees, 1986). Implantation occurs on approximately GD 6, and teratogens generally have an all-or-none effect on the organism during the preimplantation phase of development, which is considered to be approximately GD 1–7. GD 8–21 was chosen as the period of IV nicotine administration because this time includes the initial stages of neurogenesis and migration, synaptogenesis, gliogenesis, and myelination. Thus, this period represents a window of prenatal susceptibility for nicotine's teratogenic effects on neural development (Dwyer et al., 2008). Intravenous injections were delivered through the Luer-Lok injection cap of the subcutaneously implanted injection port. Following the first and second injections catheters were "post-flushed" with 0.2 ml of 0.9% physiological saline because 0.2 ml represents the approximate volume of the catheter. Post-flush of 0.2 ml of heparinized saline was used to flush the catheter and to maintain catheter patency after the third, daily nicotine injection. All IV nicotine, saline, and post-flush injections were 20 s in duration. All injections were performed during the light portion of the photoperiod, and injections were administered daily at approximately 1000, 1300, and 1600.

SURROGATE FOSTERING, LITTER COMPOSITION, AND POSTNATAL TESTING

The day of birth was considered postnatal day (PND) 0. On PND 1 litters were culled to 10 with five males and five females whenever possible. All pups were surrogate-fostered to timed-pregnant, drug naïve dams to prevent poor maternal care (Vorhees, 1986). The developmental milestones of the righting reflex, negative geotaxis, and eye opening was assessed on PND 3–5, 8–10, and 13–17, respectively. For each experiment, only one male and one female randomly selected from each litter were assigned to each treatment group (Holson and Pearce, 1992). The righting reflex was assessed by placing animals on their backs; upon releasing the animals the latency to right themselves to their stomachs was recorded. The righting reflex was assessed in blocks of three trials across three consecutive days with a maximum latency of 25 s per trial. During negative geotaxis testing animals were placed on a wire mesh grid, positioned in a 25° downward angle. The latency (30 s maximum) for animals to turn 180° to face up the slope was recorded. Negative geotaxis was measured in blocks of three trials across three consecutive days. Each animal's eyes (left and right) were checked for degree of openness across five consecutive days. The degree of openness was rated on a scale of 0–3: 0 = completely closed; 1 = any opening exposing the cornea, regardless of how small; 2 = cornea and pupil are exposed but eye lids are not fully open; 3 = fully open. All animals' weights were recorded on PND 1, 7, 14, and 21. Rats were weaned and pair housed, same sex, on PND 21.

EXPERIMENT 1: METH SELF-ADMINISTRATION

APPARATUS

Operant chambers (ENV-008; Med-Associates, St. Albans, VT, USA), housed within sound-attenuating enclosures, were controlled by Med-PC computer interface software. The front and back panels of the chamber were stainless steel and the sides and top were constructed of polycarbonate. The front panel of the chamber allowed access to a recessed food dipper (ENV-202M) through a 5-cm × 5-cm opening. Two retractable metal levers (ENV-112BM) on either side of the opening were located 7.3 cm above a metal grid floor. A dipper equipped with a 0.1-ml cup attached to the end of the dipper arm was raised into the food receptacle, which allowed access to sucrose following the completion of a response requirement. A 28-V white cue light, 3 cm in diameter, located above each response lever was used to signal time-outs. An infrared sensor (ENV-254-CB) was used to detect head entries into the food receptacle. During drug self-administration sessions a syringe pump (PHM-100) was used to deliver intravenous infusions through a water-tight swivel (PHM-115).

SUCROSE-MAINTAINED RESPONDING: PRELIMINARY TRAINING

Prenatal nicotine ($n = 13$ males; $n = 9$ females) and PS ($n = 9$ males; $n = 7$ females) offspring were food restricted in order to maintain 85% of free-feeding weight for 3 days prior to the beginning of dipper training. Dipper training and autoshaping were conducted according to previous research (Reichel et al., 2008; Lacy et al., 2012). Sucrose (26% w/v), was used as the reinforcer during initial training sessions. Offspring were between 65 and 75 days of age at the beginning of preliminary training.

SUCROSE-MAINTAINED RESPONDING: FR TRAINING

Both levers were presented in the chamber, and rats learned to respond for continuous reinforcement during 30-min sessions. A response on the active lever resulted in 4 s of access to sucrose, whereas responding on the inactive lever was recorded, but not reinforced. After the first day on continuous reinforcement, food was returned to the animal's home cage allowing *ad libitum* access for the remainder of the experiment. Animals were maintained on the FR-1 schedule for 1 day and then transitioned to a FR-3 for 2 days. Stable responding was operationally defined as greater than 2:1 ratio of active to inactive responses, and a minimum of 50 reinforcements by the second day of FR-3.

SURGERY: INDWELLING, EXTERIORIZED CATHETERS

Following acquisition of operant responding for sucrose reward animals were allowed to free-feed for a minimum of 5 days before

undergoing catheterization surgery. Animals were anesthetized with ketamine (100 mg/kg/ml) and diazepam (5 mg/kg/ml) then implanted with a catheter into the right jugular vein. The catheter exited through a dental acrylic head mount. The head mounts are secured to the skull with metal screws according to the methods of Harrod et al. (2001). Animals were allowed to recover from surgery for a minimum of 6 days during which time their catheters were flushed with heparinized saline (0.2%). Animals were weighed daily in order to monitor post-operative recovery.

METH SELF-ADMINISTRATION: FR TRAINING

During 60-min sessions, both the right and left levers were presented in the operant chamber. For FR-1 training each response on the active lever resulted in a 5.9-s, intravenous infusion of METH (0.05 mg/kg/infusion), which delivered approximately 0.1 ml of solution. Responding on the inactive lever was recorded but not reinforced. A 20-s signaled time-out, i.e., the illumination of both cue lights, was initiated with the onset of the syringe pump. Animals were not reinforced for responding on either lever during the signaled time-out. Animals were maintained on the FR-1 schedule for a minimum of 5 days. Once stable responding was exhibited, animals were transitioned to a FR-3 schedule of reinforcement. Stable responding was operationally defined as less than 20% variability of active lever responding across two consecutive sessions, greater than 2:1 ratio of active to inactive responses, and a minimum of 10 reinforcements per session. Offspring were 95–105 days old when METH self-administration began.

FR TESTING

After animals had displayed two consecutive days of stable responding on a FR-3 schedule the rats began testing on the same schedule for five different concentrations of METH. The concentrations were 0.0005, 0.0025, 0.005, 0.025, and 0.1 mg/kg/infusion, which were presented according to a Latin-square design. Animals returned to a FR-3 schedule and responded for 0.05 mg/kg/infusion for a minimum of 1 day between each FR testing session, and these were referred to as maintenance days. All sessions were a maximum of 1 h in length.

EXPERIMENT 2: METH-INDUCED CTA

MATERIALS

During testing sessions animals received access to water or sucrose via 100 ml graduated cylinders equipped with a #6.5 rubber stopper and 2.5″ straight drinking tube on the home cage (OT-100; Ancare, Bellmore, NY, USA). During the water recovery period standard drinking bottles were used.

PROCEDURE

Acquisition

Conditioned taste aversion was assessed using 124 adult animals, 65 males, and 59 females. Offspring were between 92 and 114 days of age at the beginning of the experiment. Two doses of METH (0.3 and 0.56 mg/kg/ml) were injected subcutaneously (sc) as the US. Control animals were injected with saline (Sal; sc) rather than METH. Animals were assigned to one of the following six groups: PS-0.3 (male, $n = 11$; female, $n = 9$), PN-0.3 (male, $n = 11$; female, $n = 11$), PS-0.56 (male, $n = 11$; female, $n = 9$), PN-0.56 (male, $n = 11$; female, $n = 11$), PS-Sal (male, $n = 10$; female, $n = 9$), PN-Sal (male, $n = 11$; female, $n = 10$). All rats received water restriction for 4 days prior to conditioning. During water restriction rats were given access to water for 15 min/day in 100 ml graduated cylinders. Conditioning began on day 5. Animals were presented with the saccharin (0.1% w/v) CS for 30 min, and were then injected with either METH 0.3 or 0.56 mg/kg as the US or Sal. On day 6, animals were given access to regular drinking water in standard bottles for 30 min, and no injections were administered following water consumption. This procedure was repeated three times in total. Thus, rats were conditioned on days 5, 7, and 9, and water recovery days occurred on days 6, 8, and 10. CS-US pairings occurred between 1400 and 1800. The dependent measure was the amount saccharin consumed on each of the three conditioning days. Saccharin consumption was weight-corrected [(ml consumed)/(body weight in grams)] because of the baseline sex difference in body weight.

Extinction

Repeated exposure to the CS in the absence of the US will result in a progressive attenuation of the CR. In order to generate extinction curves for the PS and PN rats, all animals were administered a daily, two-bottle test on days 11–24. During two-bottle testing, the bottles contained either water or saccharin, and the bottles were placed on the cage in a balanced manner across groups. Animals were given access to the bottles for 30 min. Preference scores were derived by dividing the amount of saccharin consumed by the combined amount of saccharin and water consumed. The scores derived from this measure range from 0 to 1.0. Scores greater than 0.5 indicate a preference for saccharin, and scores below 0.5 show a preference for water. Males and females from each prenatal treatment and drug treatment were represented during each of the conditioning and testing sessions. Extinction tests occurred between 1400 and 1800.

DRUGS

Nicotine hydrogen tartrate and METH hydrochloride were acquired from Sigma-Aldrich Pharmaceuticals (St. Louis, MO, USA). Nicotine (base weight) and METH (salt weight) were dissolved in physiological saline (0.9%; Hospira, Inc., Lake Forest, IL, USA). The pH of the nicotine solution was neutralized to approximately 7.0 with NaOH. Heparin (APP Pharmaceuticals, Schaumburg, IL, USA) was added to saline and the heparinized saline solution (2.5%) was used to flush the IV catheters.

DATA ANALYSIS

General: litter parameters

The between-subjects factors for the litter parameter analyzes were Sex and Prenatal treatment (PN or PS). The within-subjects factors were PND and GD. A one-way analysis of variance (ANOVA) was conducted for the total number of pups born to PN and PS dams. A Sex × Prenatal Treatment factorial ANOVA was used to analyze the ratio of males to females born to PN and PS dams. A Sex × Prenatal Treatment × PND mixed-factorial ANOVA was conducted for the pup weight gain, righting reflex, negative geotaxis, and eye opening data. A Prenatal Treatment × GD mixed-factorial ANOVA determined if there were differences between PS and PN dams on the measure of maternal weight gain.

Experiment 1: METH self-administration

The dependent measure for the METH self-administration study was active lever presses. Analysis of the FR-3 testing data utilized a $2 \times 2 \times 5$ mixed-factorial ANOVA. The between-subjects factors were Prenatal Treatment and Sex, and the within-subjects factor was Dose (METH dose; 0.0005, 0.0025, 0.005, 0.025, 0.1 mg/kg/infusion). The same factorial ANOVA was also conducted on the inactive lever data. These analyses were considered significant at $p < 0.05$.

Experiment 2: METH-induced CTA

The acquisition data were analyzed with a $2 \times 2 \times 3 \times 3$ mixed-factorial ANOVA. The between-subjects factors were Prenatal Treatment, Sex, and Dose (0, 0.3, 0.56 mg/kg METH). The within-subjects factor was Day, which represents the 3-days of conditioning trials. The dependent measure for the acquisition data was saccharin consumption (ml/g). Because there was a significant main effect of sex on the weight-corrected data, which indicates that females consumed more saccharin relative to males, the percent of control data were analyzed with a $2 \times 2 \times 3 \times 2$ mixed-factorial ANOVA (the factor of dose was reduced to two) in order to account for the baseline sex difference in saccharin consumption.

A $2 \times 2 \times 3 \times 14$ mixed-factorial ANOVA was used to analyze the two-bottle, extinction data. The between-subjects factors were Prenatal Treatment, Sex, and Dose. The within-subjects factor was Day. For the extinction data, the dependent measure was saccharin preference score [(saccharin)/(saccharin + water)]. Following a significant Dose × Day interaction, dependent *t*-tests were conducted on days 1 and 14 of extinction to confirm if animals in the SAL, METH 0.3, and METH 0.56 groups exhibited a change in preference, i.e., extinction, from day 1 to day 14. Dunnett's tests (Bonferroni correction), which compare treatment groups to a single control, were conducted on day 14 data to determine if the preference scores exhibited by the METH 0.3 and METH 0.56 groups differed from controls. These analyses are important to determine if treatment groups exhibited full or partial extinction. All analyzes were considered significant at $p < 0.05$.

RESULTS

LITTER PARAMETERS

Data gathered from the PN and PS-exposed pups revealed no significant effect of prenatal treatment on the number of pups born, the ratio of male vs. female pups, righting reflex, negative geotaxis, or eye opening (data not shown). Further, there was no significant effect of Prenatal Treatment on dam weight gain (**Figure 1A**) or pup weight gain (**Figure 1B**). These findings indicate that PN exposure did not disrupt postnatal development according to the ontogenetic measures used in the present study.

EXPERIMENT 1: *METH SELF-ADMINISTRATION*

A $2 \times 2 \times 5$ mixed-factorial ANOVA revealed main effects of Dose [$F(1, 34) = 43.6$, $p < 0.001$] and Prenatal Treatment [$F(1, 34) = 4.3$, $p < 0.05$], and a significant Dose × Prenatal Treatment interaction [$F(1, 34) = 4.9$, $p < 0.05$]. Sex was not a significant factor in the analyses. The METH self-administration data are presented as two separate dose-response curves in **Figure 2**. As can be seen in **Figure 2**, varying the unit dose of IV METH produced the standard "U" shaped curve for the PN and PS groups, which is commonly observed when animals self-administer psychostimulant drugs, such as amphetamines or cocaine (Yokel, 1987). Furthermore, the PS and PN groups exhibited similar means of active lever presses when allowed to respond for the lowest and highest unit doses of IV METH tested. However, PS rats clearly showed peak responding at the 0.025-mg/kg/infusion dose, whereas the PN animals exhibited peak responding between the 0.005- and 0.025-mg/kg/infusion doses. The main effect of Prenatal Treatment indicates that PN animals responded more for METH compared to the PS rats. The significant Dose × Prenatal Treatment interaction indicates that the PN dose-response curve shifted to the left. Analysis of inactive lever responding revealed a significant effect of Dose [$F(1, 34) = 26.9$, $p < 0.001$] and no other significant effects were found. The means (±SEM) for inactive lever responding were 10.0 (±1.6), 8.9 (±1.3), 6.5 (±0.9), 5.0 (±0.8), and 3.9 (±0.6; data not shown) for the 0.0005, 0.0025, 0.005, 0.025, and 1.0 doses, respectively. The main effect of dose shows that rats responded more on the inactive lever when the dose was 0.0005

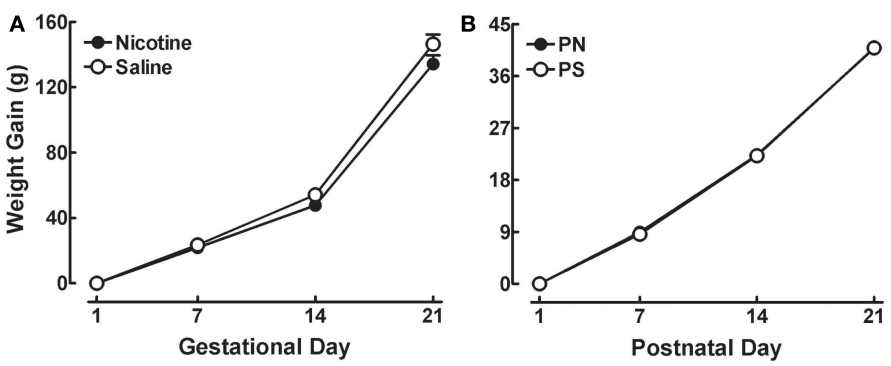

FIGURE 1 | (A) Mean (±SEM) weight gain data for the Saline and Nicotine dams across gestation. **(B)** Mean (±SEM) weight gain data for the PN and PS pups across PND 1–21.

and responding on this lever decreased as the unit dose of METH increased.

These results indicate that the PN rats exhibited a leftward shift in the dose-response curve relative to rats in the PS treatment group. This indicates that PN rats were more sensitive to the reinforcing effects of IV METH compared to the PS animals.

EXPERIMENT 2: METH-INDUCED CTA

Acquisition of CTA

A Sex × Prenatal Treatment × Dose × Day (2 × 2 × 3 × 3) mixed-factorial ANOVA was conducted on the weight-corrected data. The analysis revealed a main effect of Sex: [$F(1, 224) = 114.9$, $p < 0.001$], which indicates that females consumed more saccharin relative to males. According to this analysis females consumed an average of 0.06 ml/g of saccharin (SEM = 0.001), whereas males consumed an average of 0.04 ml/g of saccharin (SEM = 0.001; data not shown). Percent of control values were calculated, and a Sex × Prenatal Treatment × Dose × Day (2 × 2 × 2 × 3) mixed-factorial ANOVA was conducted on these data in order to adequately assess potential sex differences in CTA. The analysis revealed no significant effects of Sex or Prenatal Treatment. **Figure 3** shows the acquisition curves (% of control) for PN and PS groups injected with METH 0.3 or 0.56 mg/kg by conditioning trials. The PN and PS groups represent both males and females; however, the factors of Prenatal Treatment are represented on **Figure 3** because prenatal treatment was the focus of the experiment. The significant main effect of Day shows that animals treated with the METH US exhibited decreased saccharin consumption as a function of conditioning day [$F(2, 152) = 484.8$, $p < 0.001$] and the main effect of Dose indicates that overall the magnitude of CTA was greater in the METH 0.56 mg/kg group [means (±SEM) = 0.667 (±0.018)], relative to the METH 0.3 mg/kg group [0.798 (±0.018); $F(1, 76) = 26.9$, $p < 0.001$]. Moreover, repeated saccharin-METH pairings produced a progressive increase in the magnitude of CTA, and greater avoidance behavior was observed from animals injected with the higher dose of METH, according to the significant Day × Dose interaction [$F(2, 152) = 20.5$, $p < 0.001$].

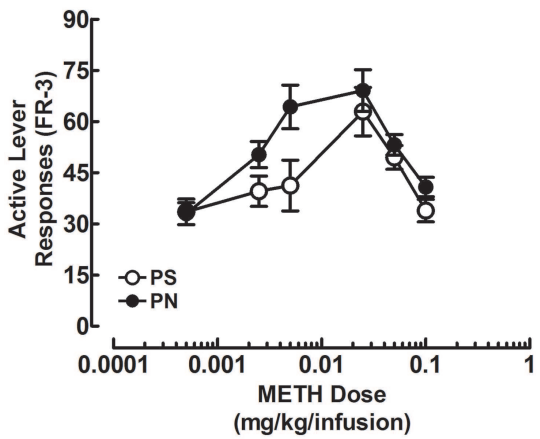

FIGURE 2 | Mean (±SEM) active lever responses for the PS and PN groups are presented for the METH doses 0.0005, 0.0025, 0.005, 0.025, 0.05, and 0.1 mg/kg/injection according to a $\log_{10}$ scale. Data from the training dose, 0.05 mg/kg/injection, is shown on the graph as a point of reference, but was not included in the ANOVA. This point represents the average responding for 0.05 mg/kg/infusion METH from the day before testing with the 0.0005- to 0.1-mg/kg/infusion doses. PN animals showed a leftward shift of the dose-response curve relative to the PS rats [Prenatal Treatment × Dose interaction; $F(1, 34) = 4.9$, $p < 0.05$].

Extinction of CTA

A 2 × 2 × 3 × 14 mixed-factorial ANOVA was conducted on the extinction data. There were no significant main effects or interactions with the factors of Sex or Prenatal Treatment. **Figure 4** shows the saccharin preference data for the PN and PS groups injected with SAL or METH 0.3 or 0.56 mg/kg US as a function of extinction trials. The PN and PS groups represent both males and females; however, the factors of Prenatal Treatment are represented on **Figure 4** because prenatal treatment was the focus of the study. Scores above 0.5 indicate a preference for saccharin, whereas scores below 0.5 indicate a preference for water.

According to the significant main effect of Dose [$F(2, 112) = 103.8$, $p < 0.001$], different magnitudes of CTA were exhibited by animals treated with SAL, METH 0.3, and METH 0.56 mg/kg. Thus, overall, rats treated with SAL or METH 0.3 mg/kg exhibited saccharin preference of 0.86 (±0.03) and 0.58 (±0.03), respectively; whereas the METH 0.56 mg/kg group showed an overall water preference 0.29 (±0.03). The main effect of Day [$F(13, 1456) = 38.9$, $p < 0.001$] shows that preference scores changed following repeated, non-reinforced exposure to the saccharin/water test stimuli; and the Day × Dose interaction [$F(26, 1456) = 7.5$, $p < 0.001$] indicates that the preference scores changed across testing days and was dependent on whether the conditioning dose was 0.3 or 0.56 mg/kg.

In order to assess responding following repeated, non-reinforced exposure to the CS, within-subjects comparisons

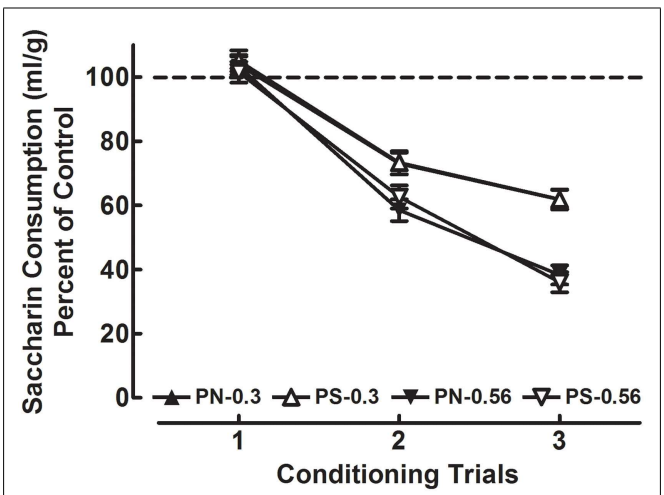

FIGURE 3 | Mean (±SEM) percent of control saccharin consumption (ml/g) data from the acquisition phase of the experiment. The CS was saccharin (0.1%, w/v) and the US was METH (SAL, 0.3 or 0.56 mg/kg). Data are shown as percent of control for the PS and PN animals treated with METH across conditioning days 1–3. There were no significant effects or interactions with the factor of Prenatal Treatment or Sex.

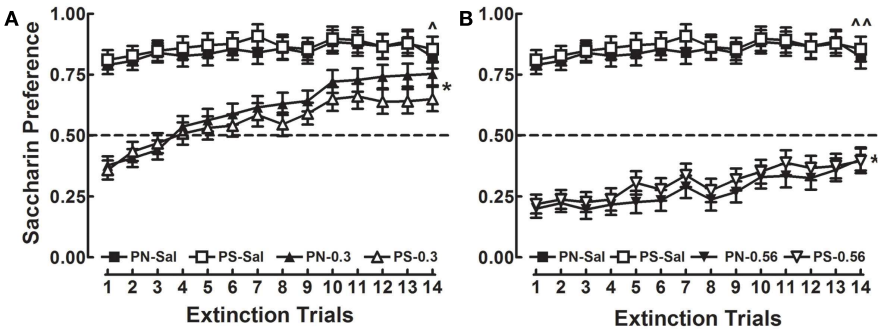

FIGURE 4 | Mean (±SEM) preference scores for the 14 consecutive two-bottle extinction tests are presented for the PS and PN animals that were treated with either SAL or 0.3 mg/kg METH (A) or SAL or 0.56 mg/kg METH (B). Scores above 0.5 indicate a preference for saccharin and scores below 0.5 indicate a preference for water. There were no significant effects or interactions with the factor of Prenatal Treatment or Sex. Significant differences between days 1 and 14 are indicated by * ($p < 0.001$). Significant differences between SAL and the 0.3- and 0.56-mg/kg METH groups are indicated by ^ ($p < 0.05$) and ^^ ($p < 0.001$).

between day 1 and 14 were conducted for the SAL, METH 0.3, and METH 0.56 groups, as described by the Day × Dose interaction. Animals in the SAL group showed a consistent preference for saccharin between testing days 1 and 14 [$t(39) = 1.49$, $p > 0.05$]. Rats treated with METH 0.3 initially showed a preference for water on day 1, and repeated exposure to the CS/water resulted in extinction, which is represented as a saccharin preference on day 14 [$t(41) = 9.37$, $p < 0.001$]. The change in responding represents a 91% increase in the preference score. Animals in the METH 0.56 group exhibited a preference for water on day 1, and despite a similar 91% increase in preference score, these animals maintained an overall preference water on day 14 [$t(41) = 4.62$, $p < 0.001$; **Figure 4**]. Dunnette's tests were conducted for data from the three groups on day 14 to determine if either the METH 0.3 or 0.56 groups were different than controls on the final day of extinction training. The preference scores from both the METH 0.3 and 0.56 groups were significantly lower than the SAL group ($p < 0.05$ and $p < 0.001$, respectively). These findings show that non-reinforced presentation of the CS produced extinction of the CR for both doses of the METH US and 14 days of extinction did not fully extinguish the CR relative to the SAL controls. These data indicate that PN exposure did not result in differential acquisition or extinction of METH-induced CTA when rats were conditioned and tested during adulthood.

DISCUSSION

The present experiments determined if IV PN exposure altered the reinforcing and/or aversive effects of METH in adult offspring. In experiment 1, PS and PN rats were trained to self-administer METH according to a FR-3 schedule of reinforcement. Following stable responding, rats were tested on varying doses of METH and dose-response curves were generated for both groups. Although both the PN and PS treatment groups exhibited the standard "U"-shaped dose-response curve (Yokel, 1987), PN rats exhibited more active lever responses for doses of METH, relative to controls. The leftward shift in the PN rats' dose-response curve, as indicated by a significant main effect of prenatal treatment and a prenatal treatment × dose interaction, indicates that these animals were more sensitive to the reinforcing effects of METH compared to rats in the PS group.

The enhanced sensitivity to IV METH exhibited by offspring in the present study may be related to nicotine's ability to induce transcriptional activation of genes associated with drug reward and sensitization of the mesocorticolimbic system (Zhu et al., 2007; Levine et al., 2011). In a study by Levine et al. (2011), mice were preexposed to oral nicotine or water and then were treated with either saline or cocaine. Nicotine-treated mice exhibited enhanced cocaine-induced locomotor sensitization and cocaine-mediated conditioned place preference, and increased cocaine-induced expression of accumbal FosB relative to controls that did not receive nicotine treatment. The nicotine-mediated increase in cocaine-induced FosB expression was associated with changes in histone acetylation, suggesting an epigenetic explanation of the findings (Levine et al., 2011). These and other findings suggest that PN may modulate histone acetylation of FosB during neurodevelopment, potentially leading to alterations in other transcription factors, and D_1 mediated signaling proteins (Teegarden et al., 2008). Indeed, previous research demonstrates that PN alters MAPk and PI3k signaling pathways (Wei et al., 2011) and increases levels of mRNA and protein of brain-derived neurotrophic factor throughout the mesocorticolimbic DA system (Harrod et al., 2011; Wei et al., 2011). Thus, determining the effects of IV PN on histone acetylation at FosB expression in offspring is of interest for future investigation.

Adolescent offspring of continuous PN exposure were previously reported to exhibit altered responding for IV cocaine. In that study Franke et al. (2008) investigated cocaine self-administration using two different concentrations of the reinforcer, 200 or 500 μg/kg/infusion, and focused on the acquisition of FR-1 responding using adolescent offspring. These findings are important because they demonstrate that rats treated with continuous PN acquired self-administration at different concentrations of cocaine: PN-treated rats acquired stable responding when the concentration was 500, but not 200 μg/kg/infusion, whereas the opposite relationship was observed for controls. Thus, these data show that PN rats required a concentration of cocaine that was 2.5 times higher than the dose needed for controls to acquire the

response. Thus, the Franke et al. (2008) experiment suggests that during the developmental period of adolescence, the PN animals were less sensitive to the stimulus properties of IV cocaine than PS offspring.

The present experiment focused on changes in the relative sensitivity to IV METH after adult offspring acquired stable responding on a FR-3 schedule of reinforcement. Thus, offspring were tested intermittently with various novel doses of the reinforcer after maintenance of the CR was demonstrated. That adult PN rats self-administered a lower dose of METH relative to controls demonstrates that PN exposure alters the stimulus properties of IV METH. Moreover, these effects of IV PN exposure appear to endure, as offspring did not receive exposure to any drug between birth and METH self-administration, which began during adulthood. This is an important point because nicotine exposure during adulthood can alter gene expression and this can lead to an altered response to other drugs of abuse, such as cocaine (Levine et al., 2011). Further experiments need to determine if PS and PN animals show differences in METH self-administration when progressive-ratio schedules of reinforcement are used (Richardson and Roberts, 1996). Such data will be necessary to determine if IV PN exposure results in motivational differences for the reinforcing effects of METH. Moreover, these findings suggest that PN exposure may alter the function of various molecular targets for METH, such as VMAT and DAT, through which this drug changes synaptic monoamine levels (Dwoskin and Crooks, 2002). Overall, PN exposure alters the appetitive stimulus properties of psychostimulant drugs like cocaine, nicotine, and METH, and these findings suggest that such alterations in the reinforcing effects of abused drugs could be one factor that underlies the increased vulnerability to drug abuse that is documented in offspring of *in utero* tobacco smoke exposure.

The aversive effects of abused drugs may play an important role in offsetting the transition from recreational drug use to chronic drug taking behavior (see Davis and Riley, 2010). Preclinical research shows that nicotine exposure during early stages of development (e.g., periadolescence) can alter learning about the aversive effects of abused drugs in adulthood (Rinker et al., 2011). Therefore it was also of interest to determine if PN exposure affects the acquisition and expression of METH-induced CTA. Experiment 2 showed that PN exposure did not alter METH-induced CTA. PN and PS animals acquired CTA in a dose-dependent manner and both treatment groups exhibited similar magnitudes of conditioned responding, as measured by the acquisition and extinction procedures. Thus, PN exposure did not alter the aversive effects of METH when measured by the CTA procedure. Further research needs to determine if a higher dose of IV PN will impact the acquisition and expression of METH-induced CTA in offspring in order to fully determine if *in utero* nicotine exposure can alter learning about the aversive effects of METH.

Low birth weight in offspring of maternal smoking is well documented (Ernst et al., 2001). These effects are produced, in part, from nicotine, because PN exposure alone produces lower birth weights in exposed rodent offspring relative to controls. This effect has been observed in animal models using the continuous, IV, and drinking water methods of nicotine exposure (e.g., Slotkin, 2004; LeSage et al., 2006; Levin et al., 2006; Schneider et al., 2010), and in general, the differences in weight are resolved with maturity. Lowering the dose of PN avoids birth weight deficits in offspring across all of the abovementioned exposure models (e.g., Franke et al., 2008; Schneider et al., 2010; Harrod et al., 2011), yet these studies report neurochemical and behavioral alterations in the offspring. The low dose, IV model of PN administration used in the present experiment has not produced altered birth weight or weight gain in offspring (Harrod et al., 2011; Lacy et al., 2011, 2012), according to the described procedures. The current findings add to a growing literature, which shows that PN exposure produces neurochemical and behavioral changes in offspring born of normal birth weight (Pauly et al., 2004; Franke et al., 2007, 2008; Schneider et al., 2010).

Taken together, the present findings indicate that IV PN exposure altered the stimulus properties of METH when an appetitive conditioning procedure was used. This finding adds to a growing literature, which shows that IV PN can alter the behavior of offspring in later stages of development (LeSage et al., 2006; Harrod et al., 2011; Lacy et al., 2011). An advantage to using the IV route of administration is that IV injection allows for 100% bioavailability of nicotine absorption and near instantaneous distribution. And, as previously mentioned, the IV route of administration closely mimics the pharmacokinetics of nicotine produced through cigarette smoke inhalation. Another advantage is that this exposure method may be used to deliver less daily amounts of nicotine relative to other exposure models (Russell and Feyerabend, 1978; Mactutus, 1989; Booze et al., 1999; Benowitz et al., 2009). Lacy et al. (2011) showed that the IV exposure method used in the present study produced deficits in prepulse inhibition of the acoustic startle response that are consistent with that induced by a continuous GN exposure model (i.e., Popke et al., 1997), for example. There are clearly disadvantages to all methods of PN exposure. A disadvantage of the present model may be that it requires direct interaction with the dam in order to administer the daily prenatal injections. On the other hand, this method affords a unique opportunity to monitor the animals' progression through pregnancy without adding what would otherwise be considered additional stress. Another disadvantage is that the present model uses nicotine to mimic maternal smoking when there are approximately 4,000 active compounds in cigarette smoke.

The present experiments indicate that offspring of IV PN exposure showed increased sensitivity to the reinforcing effects of the highly abused drug METH. This finding suggests that women who smoke a "low" number of daily cigarette during pregnancy may alter the development of motivational systems in their offspring. Increased sensitivity to the reinforcing properties of abused drugs may be one important factor that mediates the increased vulnerability toward substance abuse in the offspring of women who smoke tobacco throughout pregnancy.

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Cigarette smoking and brain regulation of energy homeostasis

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Cigarette smoking is an addictive behavior, and is the primary cause of cardiovascular and pulmonary disease, and cancer (among other diseases). Cigarette smoke contains thousands of components that may affect caloric intake and energy expenditure, although nicotine is the major addictive substance present, and has the best described actions. Nicotine exposure from cigarette smoke can change brain feeding regulation to reduce appetite via both energy homeostatic and reward mechanisms, causing a negative energy state which is characterized by reduced energy intake and increased energy expenditure that are linked to low body weight. These findings have led to the public perception that smoking is associated with weight loss. However, its effects at reducing abdominal fat mass (a predisposing factor for glucose intolerance and insulin resistance) are marginal, and its promotion of lean body mass loss in animal studies suggests a limited potential for treatment in obesity. Smoking during pregnancy puts pressure on the mother's metabolic system and is a significant contributor to adverse pregnancy outcomes. Smoking is a predictor of future risk for respiratory dysfunction, social behavioral problems, cardiovascular disease, obesity, and type-2 diabetes. Catch-up growth is normally observed in children exposed to intrauterine smoke, which has been linked to subsequent childhood obesity. Nicotine can have a profound impact on the developing fetal brain, via its ability to rapidly and fully pass the placenta. In animal studies this has been linked with abnormal hypothalamic gene expression of appetite regulators such as downregulation of NPY and POMC in the arcuate nucleus of the hypothalamus. Maternal smoking or nicotine replacement leads to unhealthy eating habits (such as junk food addiction) and other behavioral disorders in the offspring.

Keywords: smoking, nicotine, appetite regulation, reward, programming

INTRODUCTION

Cigarette smoking is the leading preventable cause of death and disability from respiratory disease. Smoking causes addiction and is negatively correlated with body weight and caloric intake; an effect which appears to be nicotine-mediated (Hajek et al., 1988). It is this action of nicotine on energy homeostasis that is attracting attention as a potential weight loss treatment during the current global obesity pandemic. However, the fat loss associated with nicotine has not been confirmed in human subjects under well-controlled experimental conditions. This review will decipher the neurophysiological mechanisms that underlie the regulation of cigarette smoking/nicotine on energy homeostasis based on both animal and human studies. The impact of maternal smoking on fetal energy homeostatic regulation will also been discussed, as there is a relatively high rate of smoking during pregnancy. Finally, whether or not nicotine is a good candidate as a weight loss treatment will be discussed.

CIGARETTE SMOKING AND WEIGHT CONTROL

Cigarette smoking is an addictive behavior with the consequences being the leading preventable cause of death and disability worldwide. It is a primary cause of cancer and cardiovascular and pulmonary disease. There are >1 billion people who smoke around the world (DeMarini, 2004), with ∼6 million deaths each year being due to tobacco/cigarette smoking-related disease; resulting in significant social and economic cost to Society (World Health Organization, 2011). It has been estimated that in less than 40 years, deaths due to smoking-related illness will rise to ∼10 million per year (DeMarini, 2004; Hussein et al., 2007).

Smoking induces a negative energy state, characterized by reduced energy intake and body weight, which has been well documented across species (Perkins, 1992; Strauss and Mir, 2001; Bellinger et al., 2003; Fulkerson and French, 2003; Chen et al., 2006, 2007, 2008). The lowered body weight has been shown to be independent of diet type, with a similar proportion of weight loss displayed in mice consuming a diet with either low or high-fat concentrations after 7 weeks of cigarette smoke exposure (Chen et al., 2007). Unfortunately, these and similar observations have led to the public perception that smoking is associated with weight loss, and it is commonly used as a weight control strategy, especially among the young, and females (Camp et al., 1993; Wiseman, 1998; Fulkerson and French, 2003). Weight gain and increased craving

for high caloric junk food on cessation of smoking without nicotine supplementation is one of the reasons given by people that prevents them from ceasing smoking (Stamford et al., 1986; Grunberg et al., 1988; Filozof et al., 2004), and this is also supported by the literature, with >75% of former smokers gaining weight after cessation (Williamson et al., 1991; Leischow et al., 1992).

Cigarette smoke contains at least 6000 components that may directly or indirectly affect caloric intake and energy expenditure. Nicotine, the major addictive substance within cigarette smoke, is the best described for its suppressive effects on body weight and appetite in both humans and animal models (Wager-Srdar et al., 1984; Grunberg et al., 1986; Bellinger et al., 2003). Furthermore, cigarette smoke stimulates the inflammatory response associated with elevated circulating levels of inflammatory cytokines, such as tumor necrosis factor α and interleukin 6, which are associated with the development of disease states related to smoking (Fernandez-Real et al., 2003). These cytokines have been shown to inhibit appetite and affect lipid metabolism (Langhans and Hrupka, 1999; Jansson et al., 2003). Overall, studies using cigarette smoke exposure have improved insight into the effects of cigarette smoking-related anorexia and weight loss.

An important question that arises from such studies is whether lower caloric intake is the main contributor to the generally lower body weight in smokers. This question can be answered by the use of pair-fed animals, which receive the same amount of food as that consumed by smoke-exposed litter-mates. According to the results of such studies, the weight loss effects of cigarette smoke exposure were not only due to the predicted reduction in energy intake, but also to an enhanced capacity for energy expenditure (Chen et al., 2006, 2008). Increased energy expenditure and thermogenesis can occur when the proton gradient of the inner mitochondrial membrane dissipates; a state which occurs via the action of mitochondrial carrier proteins termed uncoupling proteins (UCPs; Dalgaard and Pedersen, 2001). Uncoupling of the mitochondrial proton gradient is thought to be important for the maintenance of cellular respiration, activation of substrate oxidation, and prevention of the generation of reactive oxygen species (Lee et al., 1999). There are several homologs of UCPs including UCP1, which, when active in brown fat is responsible for non-shivering thermogenesis in newborn humans, in cold acclimatization, and hibernating mammals (Cannon and Nedergaard, 2004). In contrast, UCP3 is implicated in the regulation of shivering and other forms of thermogenesis, mitochondrial fatty acid transport, and basal metabolic rate (Samec et al., 1998; Argyropoulos and Harper, 2002; Schrauwen and Hesselink, 2003). Fasting or chronic food restriction normally results in the downregulation of UCP1 expression in brown fat (Champigny and Ricquier, 1990) while nicotine induces UCP1 mRNA expression, which likely leads to enhanced energy expenditure (Yoshida et al., 1999; Arai et al., 2001). In mice directly exposed to cigarette smoke, both UCP1 and three mRNA expression was increased compared with pair-fed animals (Chen et al., 2006, 2008), suggesting that increased energy expenditure occurred despite their reduced energy intake. This theory has also been supported by data from humans, where energy expenditure was increased by nicotine administration (Perkins et al., 1989).

CIGARETTE SMOKING AND ADIPOSITY

Although smokers are generally thought to weigh less than non-smokers, smoking is actually a predisposing factor for abdominal obesity, glucose intolerance, and insulin resistance (Canoy et al., 2005; Chen et al., 2007), which is a situation not well recognized by the general public. In a rodent model, we have shown that the reduction in fat mass after cigarette smoke exposure occurred only if the mice consumed a low-fat balanced diet. In addition, this weight loss was accompanied by lean body mass wasting, including that associated with some major organs such as liver, kidney, and skeletal muscle (Chen et al., 2005, 2006, 2008). Cigarette smoke exposure failed to cause fat loss when the mice consumed a high-fat cafeteria style diet consisting of foods such as fried potatoes, cakes, and sweet biscuits; whereas lean body mass loss became the prominent cause of weight loss in these mice (Chen et al., 2007). We speculate that this observation was due to a change of food preference induced by cigarette smoke exposure or, perhaps that the nature of the high-fat diet to induce over accumulation of fat mass, even with restricted caloric intake. In both human and animal studies, food high in refined sugar and fat is more preferred when they are exposed to cigarette smoke (Marangon et al., 1998; Chen et al., 2007). Consuming such food can increase fat mass, blood lipid levels, and glucose intolerance even when the total calorie intake does not exceed the daily requirement (Shiraev et al., 2009). In contrast, when smoke-exposed mice consume a high-fat diet, they consume twice the energy of the recommended daily requirement (Chen et al., 2007). Thus, we can speculate that adiposity induced by consumption of a high-fat diet, together with the loss of lean body mass found exclusively after cigarette smoke exposure may increase the risk of metabolic disorders.

In fact, both active and passive smoking contribute to glucose intolerance and insulin resistance, leading to type-2 diabetes; and smoking cessation has been demonstrated to improve insulin sensitivity (Facchini et al., 1992; Eliasson et al., 1997). It has been suggested that insulin resistance among smokers may be due to the direct impact of nicotine, carbon monoxide, or other agents in the tobacco smoke (Facchini et al., 1992). Nicotine infusion stimulates lipolysis to increase triglyceride levels in both human and animal studies (Sztalryd et al., 1996; Andersson and Arner, 2001), while hyperlipidemia is strongly associated with the onset of insulin resistance (Stannard and Johnson, 2004). Anorexia developed in long-term smokers also contributes to muscle wasting, especially in those with chronic obstructive pulmonary disease (Morrison et al., 1988; Jagoe and Engelen, 2003). Skeletal muscle is one of the major sites for insulin-dependent glucose deposition when blood glucose rises. Thus, in smokers, the reduction in muscle mass can directly impair systemic glucose uptake, contributing to postprandial hyperglycemia, and an elevated risk of developing type-2 diabetes. Vascular changes associated with prolonged smoking may also lead to reduced blood flow to skeletal muscle and decreased insulin-mediated glucose uptake (Facchini et al., 1992).

NEUROLOGICAL MECHANISMS UNDERLYING SUPPRESSED APPETITE

CLASSICAL FEEDING REGULATORS

The reduction in energy intake associated with smoking shows a relationship to the effects of several brain appetite regulators, and

indeed, nicotinic receptors have been demonstrated in the appetite regulating area of the hypothalamus (Jo et al., 2002). The most widely studied appetite regulator is neuropeptide Y (NPY), a 36 amino acid peptide. NPY is a member of the pancreatic polypeptide family, and is abundant throughout the central nervous system and the periphery (Tatemoto et al., 1982; Allen et al., 1983). NPY is a powerful neurochemical stimulator of feeding in many species (Vettor et al., 1994; Raposinho et al., 2001), with its levels reflecting the nutritional status of the body, and contributing to the long-term regulation of energy homeostasis. Administration of NPY into different brain regions, including the hypothalamus, frontal cortex, hindbrain, and hippocampus, induces hyperphagia (even in a satiated state), decreased sympathetic activity and thermogenesis, increased fat deposition, and promotion of weight gain and obesity (Clark et al., 1984; Billington et al., 1991; Egawa et al., 1991; Raposinho et al., 2001).

In studies of a mouse model of cigarette smoke exposure, the hypothalamic NPY concentration was significantly suppressed by smoke exposure, compared with food restriction (pair-feeding; Chen et al., 2006, 2008). This effect appears to be predominately nicotine-mediated, as a similar suppression of NPY has been observed in nicotine-treated animals (Jo et al., 2002). Physiologically, the decreased hypothalamic NPY levels can upregulate the expression of orexigenic NPY receptors. However, the hypothalamic density of the NPY Y_1 receptor is reduced by chronic nicotine treatment (Kane et al., 2001). Thus, it is possible that a voluntary reduction in energy intake in smokers can be attributed to suppressed NPY signaling in both the presynaptic production of the peptide and at the postsynaptic receptor level. This inhibitory effect of nicotine on appetite may be an important clue for therapy development for the treatment of obesity. This is of significant relevance, as clinical trials targeting NPY pathways have failed in obese patients due to redundancy in the mechanisms regulating energy homeostasis.

Neuropeptide Y is not the only neuropeptide in the central nerve system that can regulate appetite and energy balance. Agouti-related protein (AgRP) is another potent orexigenic molecule, which co-localizes with NPY in hypothalamic neurons (Hahn et al., 1998). In addition, there are also melanocortins, including adrenocorticotropin and melanocyte-stimulating hormones (MSH), which are peptide cleavage products of proopiomelanocortin (POMC) and exert their effects by binding to the melanocortin receptors (MCRs). The melanocortin system is thought to be one of the most important pathways involved in food intake and energy regulation, with mutations contributing to ∼4% of genetic obesity in humans (Horvath et al., 2004). Neurons expressing orexigenic NPY and AgRP cooperate with neurons expressing anorexigenic POMC and cocaine-amphetamine-regulated transcript (CART). In the diet-induced obese mouse, when hypothalamic NPY mRNA expression was reduced, AgRP and POMC mRNA were also downregulated (Lin et al., 2000; Wang et al., 2002). This suggests that the anorexigenic neurons containing POMC respond synchronously with orexigenic neurons to maintain the balance between orexigenic and anorexigenic neuropeptides. However, in nicotine-treated mice, the hypothalamic level of CART and POMC derived α-MSH has been shown to be increased (Marty et al., 1985; Kramer et al., 2007), in the face of suppression of NPY and AgRP levels (Chen et al., 2006; Martínez de Morentin et al., 2012). In addition, it has been shown that nicotine withdrawal is linked to increased hypothalamic NPY and AgRP, although with reduced UCP3 expression (Fornari et al., 2006) resulting in an increased drive to eat, and reduced capacity for energy expenditure.

PSYCHOLOGICAL REGULATORS

Feeding is not only controlled by homeostatic mechanisms, which theoretically would allow an individual to maintain an ideal body weight in the long term. Feeding is also controlled by brain reward systems and psychological states, which reinforce the motives for excessive eating without homeostatic value (Saper et al., 2002); namely, those independent of energy expenditure. The consumption of highly palatable foods is now considered to be an addictive behavior (Heilig et al., 1989). In this respect, food and nicotine addiction may share the same central pathways. Addictive eating behavior has been suggested to be predominantly controlled by the interactions between the classical "feeding center" in the lateral hypothalamus and the nucleus accumbens within the mesolimbic system, and coordination between the neurotransmitters, such as dopamine, serotonin, and the opioid system (Saper et al., 2002). Nicotine administration releases dopamine in many brain regions involved in reward, such as the mesolimbic area, the corpus striatum, the frontal cortex, and ventral tegmental area in the brain stem (Gilbert et al., 1989; Benowitz, 2010). Increased brain release of serotonin and endogenous opioid peptides, as well as the upregulation of opioid receptors, have also been reported in various animals models following nicotine administration (Marty et al., 1985; Martínez de Morentin et al., 2012). Eating, especially binge eating, is considered to be a physiological reaction to counteract stress in some individuals (Polivy et al., 1994). Nicotine has been shown to reduce anxiety in a dose-dependent manner (Gilbert et al., 1989; Pomerleau and Pomerleau, 2007), which may also overpower the desire to eat, in addition to its suppressive ability of central orexigenic pathways. Nicotine withdrawal can cause anxiety and stress (Picciotto et al., 2002), and both can serve as powerful incentives for former smokers to either overeat or smoke again.

Tolerance due to chronic nicotine use may potentially affect its activation of the brain reward pathway. To date, only the impact of nicotine tolerance on brain dopamine release is well studied, which is also site dependent (Damsma et al., 1989; Izenwasser and Cox, 1992). Nicotine tolerance is only seen in subjective mood effects, such as dizziness and confusion as reviewed by Perkins (2002). However, this tolerance may still lead to an increased demand for nicotine if it is used as an appetite suppressant.

SMOKING DURING PREGNANCY AND THE IMPACT ON OFFSPRING

Smoking during pregnancy puts physiological pressure on the mother's metabolic system and is a significant contributor to adverse pregnancy outcomes, including miscarriage, low birth weight, preterm birth, and perinatal death (Ng et al., 2006; Nielsen et al., 2006; Raatikainen et al., 2007). Moreover, it significantly

interrupts fetal development and predicts the future risks for respiratory dysfunction, social behavioral problems, cardiovascular disease, obesity, and type-2 diabetes (Whincup et al., 1989; Orlebeke et al., 1999; Stocks and Dezateux, 2003; Burke et al., 2004; Al Mamun et al., 2006; Bruin et al., 2008b). Despite the disadvantages of maternal smoking, reports still show that ∼25–29% pregnant women smoke during pregnancy (Contal et al., 2005). Some of these processes along with the underlying neurophysiological changes are shown diagrammatically in **Figure 1**.

EFFECTS ON BODY WEIGHT AND EATING BEHAVIOR IN OFFSPRING

In Western countries, it is maternal smoking during pregnancy rather than poverty that is the major cause of low birth weight (Power and Jefferis, 2002). Even maternal obesity cannot counteract the infant growth retardation due to smoking during pregnancy (Haworth et al., 1980). Studies in humans and other primates suggest that lower birth weight associated with maternal smoking is mainly nicotine-mediated (Haworth et al., 1980; Grove et al., 2001; Collet and Beillard, 2005). However, brain weight does not appear to be affected by intrauterine nicotine exposure (Grove et al., 2001); an observation that may be due to the redistribution of nutrients to preserve brain growth, at the cost of the development of other organs such as the liver and pancreas (Ernst et al., 2001).

Catch-up growth is normally observed in children exposed to intrauterine maternal smoking, and there is evidence linking maternal smoking and childhood obesity in offspring, especially

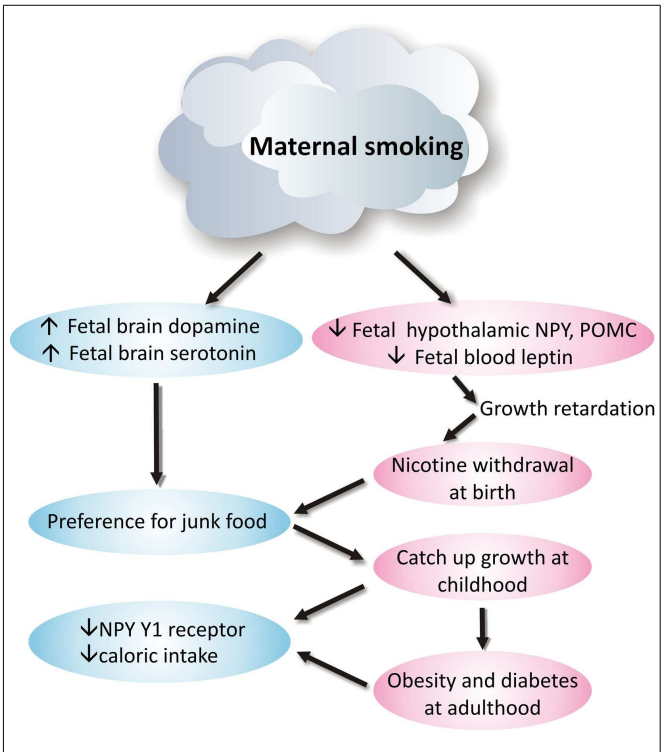

FIGURE 1 | Neurophysiological mechanism of how maternal smoking programs metabolic disorders in offspring.

those from the mothers who smoke during early pregnancy (Power and Jefferis, 2002; Al Mamun et al., 2006). It has been reported that children of mothers who smoked during pregnancy started to display an increased risk of being overweight at 5 years of age (Wideroe et al., 2003). Adolescents who are the offspring of mothers who smoked had an increased risk of being among the highest percentile for body mass index (Power and Jefferis, 2002; Al Mamun et al., 2006). Interestingly, smoking cessation after the first trimester does not appear to reduce this risk to the offspring (Toschke et al., 2003), suggesting that the first 3 months of pregnancy are critical for long-term impacts on the wellbeing of the offspring. However, children from former smoking mothers did not show increased risk of obesity (Oken et al., 2005).

Smoking mothers tend to have a shorter breastfeeding period, which deprives the offspring of the protection provided by breast milk against future eating disorders (Gilchrist et al., 2004; Mayer-Davis et al., 2006). On this basis, it can be suggested that the rapid weight gain during the early postnatal period may be due to the effect of nicotine withdrawal, in a similar manner to the increased craving for food and subsequent weight gain seen in smokers after smoking cessation (Lerman et al., 2004). Furthermore, as children also tend to copy the eating habits of their parents, this will be detrimental in the children of smokers, as smokers are more likely to choose foods low in fiber, vitamins and minerals, and high in monounsaturated fatty acids, starch, as well as sugar-sweetened soft drinks (Crawley and While, 1996; Rogers et al., 2003). Indeed, the children of smokers are more likely to be exposed to passive smoking, with ongoing detrimental effects of the chemicals in the cigarette smoke.

EFFECTS ON BRAIN ENERGY HOMEOSTATIC REGULATORS

Nicotine can have a profound impact on the developing fetal brain, via its ability to rapidly and fully pass across the placenta, with fetal concentrations ∼115% of maternal levels (Walker et al., 1999). When the fetus leaves the womb, the supply of nicotine is removed, and the impact of nicotine withdrawal can be observed in these newborns, as they show increased signs of stress and dysregulation of the hypothalamic-pituitary-adrenal axis (Huizink and Mulder, 2006). Studies in humans, other primates, and mice have observed some neuronal abnormalities relevant to feeding regulation that result from maternal smoking or exposure to nicotine (Mantzoros et al., 1997; Grove et al., 2001; Bruin et al., 2008a). However, the impact of maternal smoking during gestation on brain energy homeostatic pathways in the offspring requires further study.

Maternal smoking is clearly linked to abnormal hypothalamic gene expression of appetite regulators, with NPY and POMC gene expression in the arcuate nucleus of the hypothalamus being significantly downregulated in the newborn primate following intrauterine nicotine exposure (Grove et al., 2001); a state that may reflect an under-developed brain. This state is similar to observations in adult animals with nicotine or cigarette smoke exposure, as clarified above. Indeed, it can be suggested that without the continuing inhibition of nicotine, NPY, and POMC gene expression can rebound to that equal to an early postnatal age, leading to hyperphagia and future obesity. As yet there is no direct data to date to support this hypothesis. However, studies of mouse

models have examined the adult offspring from mothers exposed to cigarette smoke and/or those consuming a high-fat diet during the pregnancy (Chen et al., 2011). Surprisingly, despite increased adiposity in offspring from smoke-exposed mothers, their daily caloric intake was actually lower than the offspring from control mothers, regardless of postnatal diet type. Although the levels of POMC were not different between groups, NPY gene expression was only suppressed by maternal consumption of a high-fat diet, and not intrauterine smoke exposure *per se*. However, NPY Y1 receptor gene expression was significantly downregulated by both maternal smoke exposure and a high-fat diet, with this being reflected by reduced food intake in those offspring (Chen et al., 2011). In addition, other components of cigarette smoke, such as carbon monoxide and ingredients in tobacco tar, can also directly affect the fetal brain, and thereby contribute to the above changes in the fetal brain (Ernst et al., 2001). It can be suggested that at adulthood, the changes in brain appetite regulators may be an adaptation to increased adiposity, rather than a prolonged impact of intrauterine smoke exposure.

Another important appetite regulator is the adipocyte-derived hormone leptin, which is critical for the development of neurons and neural projections between hypothalamic nuclei involved in appetite control in early life (Bouret et al., 2004). In mice, a lack of leptin during the early postnatal period results in sparse neuronal projections in the hypothalamus, and later in life, an obese phenotype (Zhang et al., 1994; Chua et al., 1996; Bouret et al., 2004). Leptin supplementation during this early postnatal period can partially restore the reduced hypothalamic neural projections in the leptin-deficient *ob*/*ob* mouse, and partially reverse the hyperphagic phenotype (Bouret et al., 2004). In humans, cord blood leptin concentrations in both full-term and preterm newborns from smoking mothers are reported to be significantly decreased compared to those from non-smoking mothers (Mantzoros et al., 1997). It has been suggested that smoking might increase the production of catecholamines in the infants leading to lipolysis and fat loss, which can be associated with decreased leptin levels (Mantzoros et al., 1997; Ozkan et al., 2005), as circulating leptin levels are in relative proportion to fat mass. In a similar manner, in primates serum leptin levels are reduced by ∼50% in newborns from nicotine-treated mothers compared with those from control mothers (Grove et al., 2001). One hypothesis that may account for this observation is that reduced leptin in newborns from smoking mothers may interrupt the development of the neurons controlling energy homeostasis, contributing to unhealthy eating behavior at adulthood. As with smokers, it may be that the reward pathways override the energy homeostatic control in such offspring, resulting in a preference for junk foods. Studies of offspring from nicotine-treated animals show that dopamine receptor binding affinity is increased, despite reduced receptor density; while brain serotonin turnover was reduced, whilst its transporter was increased in such offspring (Fung and Lau, 1989; Muneoka et al., 1997, 2001). In the original studies of this topic, this finding was used to explain the abnormal social behavioral problems, such as attention deficit hyperactivity disorder or addiction, as found in offspring with intrauterine nicotine exposure. However, changes in the reward pathway may also underlie the unhealthy eating behavior.

CONCLUSION

Nicotine can change brain feeding regulation to reduce appetite via both energy homeostatic and reward mechanisms. In animal models, the effects of cigarette smoke exposure on energy homeostasis are clearly both time and dose dependent. As such, the higher the dose, the greater the reduction in caloric intake and body weight. However, the marginal effect of nicotine at reducing abdominal fat in high-fat diet fed animals may shed light on its potential application in the treatment of obesity. Maternal smoking or nicotine replacement can clearly lead to unhealthy eating habits (such as junk food addiction) and other behavioral disorders in the offspring. Thus, smoking cessation without nicotine replacement during pregnancy is recommended. Although the direct use of nicotine for fat loss in the obese is not plausible, the appetite suppressive and energy expenditure promoting effects of nicotine may still be useful. The development of nicotine analogs should be encouraged which avoid addiction, but retain the fat burning-obesity reduction effect.

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Associations of cigarette smoking and polymorphisms in brain-derived neurotrophic factor and catechol-*O*-methyltransferase with neurocognition in alcohol dependent individuals during early abstinence

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Chronic cigarette smoking and polymorphisms in brain-derived neurotrophic factor (BDNF) and catechol-*O*-methyltransferase (COMT) are associated with neurocognition in normal controls and those with various neuropsychiatric conditions. The influence of BDNF and COMT on neurocognition in alcohol dependence is unclear. The primary goal of this report was to investigate the associations of single nucleotide polymorphisms (SNPs) in BDNF Val66Met (rs6265) and COMT Val158Met (rs4680) with neurocognition in a treatment-seeking alcohol dependent cohort and determine if neurocognitive differences between non-smokers and smokers previously observed in this cohort persist when controlled for these functional SNPs. Genotyping was conducted on 70 primarily male treatment-seeking alcohol dependent participants (ALC) who completed a comprehensive neuropsychological battery after 33 ± 9 days of monitored abstinence. After controlling for COMT and BDNF genotypes, smoking ALC performed significantly worse than non-smoking ALC on the domains of auditory-verbal and visuospatial learning and memory, cognitive efficiency, general intelligence, processing speed, and global neurocognition. In smoking ALC, greater number of years of smoking over lifetime was related to poorer performance on multiple domains after controlling for genotypes and alcohol consumption. In addition, COMT Met homozygotes were superior to Val homozygotes on measures of executive skills and showed trends for higher general intelligence and visuospatial skills, while COMT Val/Met heterozygotes showed significantly better general intelligence than Val homozygotes. COMT Val homozygotes performed better than heterozygotes on auditory-verbal memory. BDNF genotype was not related to any neurocognitive domain. The findings are consistent with studies in normal controls and neuropsychiatric cohorts that reported COMT Met carriers demonstrated better performance on measures of executive skills and general intelligence. Results also indicated that the poorer performance of smoking compared to non-smoking ALC across multiple neurocognitive domains was not mediated by COMT or BDNF genotype. Overall, the findings lend support to the expanding clinical movement to make smoking cessation programs available to smokers at the inception of treatment for alcohol/substance use disorders.

Keywords: cigarette smoking, brain-derived neurotrophic factor, catechol-*O*-methyltransferase, neurocognition, alcohol dependence

INTRODUCTION

A number of premorbid and/or comorbid factors may contribute to the pattern and magnitude of neurocognitive abnormalities demonstrated by those with alcohol use disorders (AUD; Parsons and Nixon, 1993; Oscar-Berman, 2000; Sher et al., 2005; Rourke and Loberg, 2009). In our previous work assessing the neurocognitive consequences of AUD, we investigated the influence of chronic cigarette smoking, sociodemographic factors, alcohol consumption levels, as well as comorbid substance abuse, psychiatric and medical conditions (Durazzo et al., 2006, 2007b,c, 2008, 2010a). Among these variables, chronic cigarette smoking was the sole factor that consistently and robustly predicted neurocognition in our AUD participants. Specifically, chronic smoking was associated with significantly poorer performance on measures of executive skills, processing speed, and learning and memory. Additionally, longer duration of smoking over lifetime in these studies was consistently related to poorer performance on multiple domains of neurocognition after controlling for age, alcohol consumption, and other potentially mediating variables.

Human neurocognition is a complex phenotype that is a function of psychosocial, environmental, biological, and genetic factors. With respect to genetic factors, multiple studies have reported that the Val66Met single nucleotide polymorphism (SNP) of the brain-derived neurotrophic factor (BDNF; rs6265) and the Val158Met SNP of the catechol-*O*-methyltransferase (COMT; rs4680) genes are associated with several domains of neurocognitive functioning. Specifically, studies have reported that the BDNF Met allele carriers (i.e., Val/Met, Met/Met) of the BDNF demonstrated poorer verbal memory (Egan et al., 2003; Hariri et al., 2003; Dempster et al., 2005; Tan et al., 2005; Schofield et al., 2009), processing speed (Miyajima et al., 2008; Raz et al., 2009), and general intelligence (Tsai et al., 2004; Miyajima et al., 2008) in controls and individuals with various neuropsychiatric conditions (e.g., schizophrenia). The observed relationships between BDNF genotypes and neurocognition, however, were not uniform across all studies (Harris et al., 2006; Savitz et al., 2006). For COMT, studies with controls and individuals with various neuropsychiatric conditions reported that Met homozygosity was related to better performance on measures of executive skills, working memory, and general intellectual functioning. Alternately, several studies found no relationship between COMT genotype and neurocognition and some reported Val homozygosity was associated with better neurocognitive performance (for review see Savitz et al., 2006; Barnett et al., 2008; Dickinson and Elvevag, 2009; Enoch et al., 2009; Wishart et al., 2011). While the cumulative body of research appears to suggest COMT Met homozygosity is generally associated with better performance on working memory and executive function tasks, the influence of the COMT Val158Met polymorphism on neurocognition has yet to be fully elucidated (Barnett et al., 2008; Goldman et al., 2009). Overall, the majority of research on BDNF has focused on memory function and for COMT on measures of executive skills and working memory in healthy controls and individuals with neuropsychiatric disorders (e.g., schizophrenia-spectrum and bipolar disorders). We are not aware of any study that specifically investigated the association of BDNF and COMT polymorphisms with neurocognition in AUD. Therefore, it is unclear to what extent polymorphisms in BDNF and COMT are related to neurocognitive function in AUD.

The primary goal of this report was to investigate the associations of SNPs in BDNF Val66Met (rs6265), COMT Val158Met (rs4680) with neurocognition in our treatment-seeking alcohol dependent participants and determine if neurocognitive differences between non-smokers and smokers previously observed in this cohort persist when controlled for these functional SNPs. We predicted that smoking alcohol dependent participants compared to non-smokers perform significantly worse on the domains of executive skills, processing speed, and learning and memory after controlling for BDNF and COMT genotypes, alcohol consumption, age, and predicted premorbid intelligence. We also hypothesized that the inverse relationships between lifetime years of smoking and neurocognitive performance we observed in our previous studies are independent of the effects BDNF and COMT polymorphisms in the current study cohort. Finally, we predicted that BDNF Val homozygotes perform significantly better than Val/Met heterozygotes and COMT Met homozygotes show better performance than Val homozygotes on measures of executive skills, learning, memory, and processing speed, after controlling for smoking status, alcohol consumption, age, and predicted premorbid intelligence.

MATERIALS AND METHODS

PARTICIPANTS

Individuals seeking treatment for AUD ($n = 70$; four females) were recruited from the VA Medical Center Substance Abuse Day Hospital and the Kaiser Permanente Chemical Dependence Recovery Program outpatient clinics in San Francisco. All participants provided written informed consent prior to study according to the Declaration of Helsinki, and the informed consent document and procedures were approved by the University of California San Francisco and the San Francisco VA Medical Center. Participants were between the ages of 28 and 68 at the time of study and all met DSM-IV criteria for alcohol dependence (95% with physiological dependence). The alcohol dependent participants (ALC) completed a comprehensive neuropsychological assessment battery after 33 ± 9 days of monitored abstinence. Smoking ($n = 39$) and non-smoking ($n = 31$) ALC did not differ in the duration of abstinence prior to assessment. All smoking ALC were actively smoking at the time of assessment and no participant changed their cigarette consumption from the onset of abstinence to the time of assessment. Five non-smoking ALC reported a previous history of chronic smoking, with four quitting more than 8 years and one more than 3 years prior to enrollment. The performance of the former smokers was within ± 0.5 standard deviations of the non-smoking ALC group mean across neurocognitive domains. The vast majority of ALC in this study were participants in our previous research (Durazzo et al., 2008, 2010a). Demographics, indices of alcohol consumption, smoking severity, depressive and anxiety symptomatology, and frequency of medical, psychiatric, and substance use comorbidities for ALC are given in **Table 1**.

Primary inclusion criteria were current DSM-IV diagnosis of alcohol dependence or abuse, fluency in English, consumption of greater than 150 alcoholic drinks per month (one alcoholic drink equivalent = 13.6 g pure ethanol) for at least 8 years prior to enrollment for men, and consumption of greater than 80 drinks per month for at least 8 years prior to enrollment for women. Primary exclusion criteria are fully detailed in our previous work (Durazzo et al., 2004). In summary, no participant had a history of a neurologic (e.g., non-alcohol-related seizure disorder, neurodegenerative disorder, demyelinating disorder; traumatic brain injury with loss of consciousness >15 min), general medical (e.g., myocardial infarction, Type-1 diabetes, cerebrovascular accident), or psychiatric (e.g., schizophrenia-spectrum, bipolar disorder, post-traumatic stress disorder, substance dependence within 5 years prior to study) conditions known or suspected to influence neurocognition. The following comorbidities were permitted due to their high prevalence in AUD (Gilman and Abraham, 2001; Stinson et al., 2005): hepatitis C, type-2 diabetes, hypertension, unipolar mood (major depression, substance-induced mood disorder), and anxiety (generalized anxiety disorder, panic disorder). ALC who met DSM-IV criteria for current or past substance abuse were included. Current opioid replacement therapy (e.g., methadone) was exclusionary.

Table 1 | Participant demographics and clinical measures.

Measure	ALC ($n = 70$)
Age	51.0 ± 10.0
Education	14.0 ± 2.2
Days abstinent	33 ± 9
AMNART	114 ± 9
1-year average drinks/month	398 ± 206
8-year average drinks/month	314 ± 163
Lifetime average drinks/month	208 ± 100
Months of heavy drinking	259 ± 116
Age onset heavy drinking	26 ± 10
FTND	5.5 ± 2.7
Cigarette pack years	25 ± 18
Lifetime years of smoking	25 ± 12
Beck Depression Inventory	11.1 ± 9.0
STAI-trait	43.1 ± 11.0
% smokers	56
% with psychiatric comorbidity	44
% with substance comorbidity	24
% with medical comorbidity	44
GGT	44 ± 25
Prealbumin	27 ± 6

AMNART, American National Adult Reading Test; FTND, Fagerstrom Test for Nicotine Dependence; GGT, gamma glutamyltransferase, normal range 7–64; institutional units; prealbumin (proxy measure of nutritional status), normal range 18–45 mg/dl; STAI, State-Trait Anxiety Inventory; (mean ± SD).

MEDICAL, PSYCHIATRIC, SUBSTANCE, AND DRINKING HISTORY ASSESSMENT

Participant medical history was obtained from self-report and confirmed via available medical records. Participants completed the Structured Clinical Interview for DSM-IV Axis I disorders, Patient Edition, Version 2.0 (SCID-I/P; First et al., 1998), and standardized questionnaires assessing lifetime alcohol consumption (Lifetime Drinking History, LDH; Skinner and Sheu, 1982; Sobell et al., 1988) and substance use (in-house questionnaire assessing substance type, and quantity and frequency of use). From the LDH we derived average number of alcohol-containing drinks per month over 1 and 8 years prior to enrollment, average number of drinks per month over lifetime, number of lifetime years of regular drinking (i.e., consuming at least one alcoholic drink per month), number of months of heavy drinking (i.e., total number of months over lifetime of drinking in excess of 100 drinks per month), age of onset of heavy drinking and total kilograms of ethanol consumed over lifetime. Participants completed self-report measures of depressive (Beck Depression Inventory, BDI; Beck, 1978) and anxiety symptomatology (State-Trait Anxiety Inventory, form Y-2, STAI; Spielberger et al., 1977), and nicotine dependence [Fagerstrom Tolerance Test for Nicotine Dependency (FTND; Fagerstrom et al., 1991)]. The total number of cigarettes currently smoked per day, number of years of smoking at the current level and over lifetime were also recorded, and pack years [i.e., (number of cigarettes per day/20) × lifetime number of years of smoking] calculated for smoking ALC.

NEUROPSYCHOLOGICAL ASSESSMENT

Participants completed a comprehensive battery, which evaluated domains of neurocognitive function previously reported to be affected by AUD (Oscar-Berman, 2000; Rourke and Loberg, 2009) and chronic cigarette smoking (Durazzo et al., 2007a; Swan and Lessov-Schlaggar, 2007). Smoking ALC were allowed to smoke *ad libitum* prior to assessment and to take smoking breaks during testing if requested. The neurocognitive domains evaluated and the constituent measures were as follows: *Executive skills*: Short Categories Test (Wetzel and Boll, 1987), color-word portion of the Stroop Test (Golden, 1978), Trail Making Test part B (Reitan and Wolfson, 1985), Wechsler Adult Intelligence Scale 3rd Edition (WAIS-III) Similarities (Wechsler, 1997), Wisconsin Card Sorting Test-64: Computer Version 2-Research Edition (Kongs et al., 2000) non-perseverative errors, perseverative errors, and perseverative responses *General intelligence*: Ward-7 Full Scale IQ (Axelrod et al., 2001; based on WAIS-III Arithmetic, Block Design, Digit Span, Digit Symbol, Information, Picture Completion, and Similarities subtests; Wechsler, 1997). *Learning and memory*: Auditory-verbal: California Verbal Learning Test-II (Delis et al., 2000), Immediate Recall trials 1–5 (learning), Short and Long Delay Free Recall (memory). Visuospatial: Brief Visuospatial Memory Test-Revised (Benedict, 1997), Total Recall (learning), and Delayed Recall (memory). *Processing speed*: WAIS-III Digit Symbol, Stroop Color and Word (Golden, 1978), WAIS-III Symbol Search (Wechsler, 1997), Trail Making Test-A (Reitan and Wolfson, 1985). *Visuospatial skills*: WAIS-III Block Design; Luria–Nebraska Item 99 (Golden et al., 1978). *Working memory*: WAIS-III Arithmetic, WAIS-III Digit Span. *Cognitive efficiency*: this domain consisted of all tests that were timed, or in which the time to complete the task influenced the score achieved, and was calculated by averaging the individual z-scores of those measures (see below). Timed tests included the Luria–Nebraska Item 99 ratio, Stroop word, color, and color-word tests, Trails A and B and WAIS-III Arithmetic, Block Design, Digit Symbol, Picture Completion, and Symbol Search. Higher scores on these measures reflect better speed and accuracy on principally non-verbal tasks. The cognitive efficiency domain is an approximation of the concept of cognitive efficiency previously described by Glenn and Parsons (1992) and Nixon et al. (1995, 1998). Premorbid verbal intelligence was estimated with the American National Adult Reading Test (Grober and Sliwinski, 1991). For the Luria–Nebraska Item 99, the number correct (maximum possible = 8) was divided by the time required to complete the task. This ratio was used due to the low ceiling for the number of correct responses (i.e., most participants achieved a score of 6 or better), which resulted in a highly skewed and non-Gaussian distribution. The ratio of number correct to time to complete the Luria 99 was normally distributed.

Raw scores for all neurocognitive measures, except the Luria–Nebraska Item 99 ratio, were converted to age-adjusted standardized scores via the normative data accompanying the particular measure (i.e., BVMT-R, CVLT-II, Short Categories Test, Stroop Color-Word Test, WAIS-III subtests) or age and education [(WCST-64 variables; Trails A and B via Heaton Compendium Norms (Heaton et al., 1991)]. Standardized scores were transformed to z-scores for all measures. For the Luria–Nebraska Item

99 ratio, raw scores were converted to *z*-scores based on the performance of 32 non-smoking light drinking controls, as there are no published norms available for this measure. A global neurocognitive functioning score was calculated from the arithmetic mean of *z*-scores for all of the individual domains.

GENOTYPING

Genomic DNA was isolated from whole blood. The SNPs were assayed using TaqMan genotyping assays from Applied Biosystems, Foster City, CA, USA. SNP assays were performed using a reaction volume of 15 μl, which consisted of 7.5 μl of TaqMan 2X universal master mix, 0.38 μl of 20X TaqMan pre-designed SNP genotyping assay, 6.14 μl of nuclease-free water, and 1 μl genomic DNA. After PCR amplification as per manufacturer's recommendations, SNP genotypes were determined by allelic discrimination using the ABI-7500 instrument. BDNF ($\chi^2 = 0.79, p = 0.37$) and COMT ($\chi^2 = 0.01$, $p = 0.92$) were in Hardy–Weinberg equilibrium (see **Table 2**).

DATA ANALYSES

Multivariate analyses of covariance (MANCOVA) examined effects of BDNF and COMT genotypes and smoking status on the 11 domains of neurocognition (see **Table 3** for list of domains), with age, AMNART, and lifetime average drinks per month as primary covariates. In our previous work with this alcohol dependent cohort, age accounted for a significant amount of the variance in neurocognition despite the use of age-corrected norms (Durazzo et al., 2008, 2010a); therefore, age was also used as a covariate in this study. Significant MANCOVA omnibus effects ($p = 0.05$) for genotypes and smoking status were followed-up with pairwise *t*-tests. To control for the potential influence of medical (primarily hypertension and positivity for the hepatitis C antibody), psychiatric (primarily unipolar mood disorders), and substance abuse history on neurocognition, pairwise comparisons achieving statistical significance were reanalyzed using medical, psychiatric, and substance use comorbidities, individually, as additional covariates. Significance levels of all pairwise comparisons were adjusted for multiplicity of tests. Alpha levels ($p = 0.05$) for pairwise comparisons for BDNF and COMT genotypes and smoking status were adjusted for the number of neurocognitive domains evaluated (i.e., 11) and the average intercorrelation among the domains (i.e., $r = 0.55$), resulting in a corrected *p*-values of 0.017 (see Sankoh et al., 1997). Effect sizes (ES) for pairwise comparisons were calculated via Cohen's *d* (Cohen, 1988). For smoking ALC, associations (i.e., semi-partial correlations) between the 11 neurocognitive domains, genotypes, lifetime average drinks per month, and lifetime years of smoking were examined with multiple linear regression (all predictors simultaneously entered into the model). Analyses were completed with SPSS v18.0.

Table 2 | Genotype frequency for BDNF Val66Met, COMT Val158Met.

SNP	Genotype	Frequency	Percent
BDNF (rs6265)	Val/Val	47	67.1
	Val/Met	22	31.4
	Met/Met	1	1.5
COMT (rs4680)	Val/Val	21	30.0
	Val/Met	35	50.0
	Met/Met	14	20.0

SNP, single nucleotide polymorphism. All genotypes were in Hardy–Weinberg equilibrium ($\chi^2 < 0.83$, $p > 0.36$).

RESULTS

PARTICIPANT CHARACTERIZATION

Participants were 51.0 ± 10.0 years of age, had 14.0 ± 2.2 years of formal education and were abstinent for 33 ± 9 days at the time of study. Eighty percent of ALC participants were Caucasian, 13% African American, 4% Latino, 2% Native American, and 1% Pacific Islander. See **Table 1** for additional demographics and clinical measures.

SMOKING STATUS, COMT AND BDNF GENOTYPES, AND NEUROCOGNITIVE FUNCTION

Multivariate analyses of covariance indicated significant omnibus effects for smoking status [F (10, 53) = 3.18, $p < 0.003$], COMT genotype [F (20, 108) = 1.77, $p = 0.042$], age [F (10, 53) = 2.97, $p = 0.005$], and AMNART [F (10, 53) = 11.74, $p < 0.001$]. BDNF genotype and lifetime average drinks per month were not significant predictors of neurocognition. Inspection of pairwise tests across domains for BDNF Val homozygotes versus heterozygotes revealed all comparisons were $p > 0.15$, with trivial ES (all <0.16).

Pairwise comparisons indicated smoking ALC performed worse than non-smoking ALC on the following domains of functioning: auditory-verbal learning ($p < 0.001$; ES = 0.83), auditory-verbal memory ($p < 0.001$; ES = 0.87), cognitive efficiency ($p < 0.001$; ES = 0.97), general intelligence ($p < 0.001$; ES = 0.92), processing speed ($p < 0.001$; ES = 0.97), visuospatial learning ($p = 0.001$; ES = 0.75), visuospatial memory ($p = 0.007$; ES = 0.60), and global neurocognition ($p < 0.001$; ES = 1.09). Smoking ALC showed a trend for lower executive skills ($p = 0.05$;

Table 3 | Associations between neurocognitive domains (age-corrected) and lifetime years of smoking for smoking ALC (*n* = 39).

Neurocognitive domain	Lifetime years of smoking
Auditory-verbal learning	−0.39**
Auditory-verbal memory	−0.38*
Cognitive efficiency	−0.37**
Executive skills	−0.23
General intelligence	−0.27*
Processing speed	−0.30*
Visuospatial learning	−0.50**
Visuospatial memory	−0.45**
Visuospatial skills	−0.43**
Working memory	−0.16
Global neurocognition	−0.49**

**p < 0.05; **p < 0.01; all tests two-tailed. Correlations are semi-partial coefficients controlling for AMNART, lifetime average drinks per month, BDNF, and COMT genotypes.*

ES = 0.40). Controlling the above listed pairwise tests for COMT, medical, psychiatric, and substance abuse comorbidities did not appreciably alter the above *p*-values or ES for differences between smoking and non-smoking ALC.

Pairwise comparisons showed COMT Met homozygotes (i.e., Met/Met) were superior to Val homozygotes (i.e., Val/Val) on executive skills ($p = 0.013$, ES = 0.75) and showed trends for higher general intelligence ($p = 0.035$, ES = 0.61) and visuospatial skills ($p = 0.022$, ES = 0.69) than Val homozygotes. Val/Met heterozygotes demonstrated a significantly better performance on the general intelligence domain than Val homozygotes ($p = 0.014$, ES = 0.45). Val homozygotes performed significantly better than Val/Met on auditory-verbal memory ($p = 0.012$, ES = 0.65). Controlling the above listed pairwise tests for smoking status, medical, psychiatric, and substance abuse comorbidities did not alter the above reported results.

ASSOCIATIONS OF GENOTYPES WITH ALCOHOL CONSUMPTION AND LIFETIME YEARS OF SMOKING

No significant associations were observed among BDNF and COMT genotypes, alcohol consumption measures, and the 11 neurocognitive domains. For smoking ALC, higher lifetime years of smoking showed moderate to strong inverse relationships with performance on multiple neurocognitive domains after controlling for AMNART, lifetime average drinks per month, BDNF and COMT genotypes (see **Table 3**). There were no relationships between FTND score (i.e., level of nicotine dependence) and any neurocognitive domain.

DISCUSSION

The primary findings from this cohort of primarily male, treatment-seeking alcohol dependent individuals with approximately 1 month of abstinence from alcohol were as follows: (1) smoking ALC demonstrated significantly poorer performance than non-smoking ALC on multiple domains of neurocognition *after* controlling for COMT and BDNF genotypes and medical, psychiatric, and substance abuse comorbidities; (2) in smoking ALC, greater number of lifetime years of smoking was associated with worse performance on multiple neurocognitive domains; (3) COMT genotype was significantly associated with measures of executive skills, general intelligence, and visuospatial memory; and (4) the BDNF Val66Met polymorphism was not a significant predictor of any neurocognitive domain.

Chronic cigarette smoking in this cohort of alcohol dependent individuals in early recovery was a robust predictor of performance in multiple domains of neurocognition after controlling for BDNF and COMT genotypes, lifetime alcohol consumption, age, and AMNART. The pattern of inferior performance of smoking ALC relative to non-smoking ALC and the moderate to strong ES for the group differences are consistent with our previous research (Durazzo et al., 2008, 2010a) as well as with findings from other studies (e.g., Glass et al., 2006, 2009). Additionally, in smoking ALC, the relationships of greater number of years of lifetime smoking to age-adjusted scores on multiple neurocognitive domains remained significant and robust after controlling for BDNF and COMT, lifetime alcohol consumption, and comorbid conditions. Taken together, this suggests that the inferior performance of smoking compared to non-smoking ALC and the moderate to strong associations of lifetime years of smoking with neurocognition in smoking ALC were not mediated by the SNPs investigated, cumulative amount of alcohol consumed over lifetime, or conditions that are highly comorbid with AUD.

When assessing the effects of chronic cigarette smoking on neurocognition, it is important to distinguish between the effects of acute ingestion, metabolism and withdrawal of nicotine, and the influence of chronic exposure to the multitude of noxious compounds contained in cigarette smoke. Acute nicotine administration has been found to transiently improve some areas of neurocognition in healthy non-smokers and individuals with attention deficit hyperactivity disorder and schizophrenia-spectrum disorders, predominantly on measures of sustained attention and working memory (Rezvani and Levin, 2001; Sacco et al., 2004; Mansvelder et al., 2006). Acute nicotine administration in nicotine deprived smokers is associated with improved cognitive task performance (Mendrek et al., 2006; Parrott, 2006), whereas several studies report decrements in neurocognitive performance with nicotine administration to non-smokers (see Mansvelder et al., 2006 for review). A recent meta-analysis conducted by Heishman et al. (2010) suggests that acute smoking or nicotine consumption, independent of withdrawal effects, are associated with enhanced performance in the following domains of function: fine motor skills, alerting attention accuracy and response time, orienting attention reaction time, short-term episodic memory accuracy, and working memory reaction time (but not accuracy). There is limited placebo controlled research assessing the effects of acute nicotine administration in AUD. In alcohol dependent smokers with 40 ± 17 days of abstinence, a high acute nicotine dose administered via transdermal patch (14 and 21 mg for females and males, respectively), was related to greater accuracy on a measure of vigilance and working memory than a low nicotine dose (7 mg; Boissoneault et al., 2011), but neither the high nor the low nicotine dose influenced immediate or delayed auditory-verbal memory performance (Gilbertson et al., 2011). Greater pack years (a composite measure of smoking intensity and chronicity), was related to longer reaction times and lower accuracy on the vigilance and working memory task (Boissoneault et al., 2011). Similarly, in community-based samples of men with a lifetime history of alcohol dependence, higher pack years were inversely related to measures of cognitive proficiency and general intelligence (Glass et al., 2006) and both smoking and alcoholism severities were inversely related to executive function (Glass et al., 2009). In this study, longer lifetime smoking duration was associated with poorer performance on multiple neurocognitive domains, which is consistent with the findings for pack years in the above studies. sALC in this study were allowed to smoke *ad libitum* prior to assessment and to take smoke breaks during the assessment. The plasma half-life of nicotine is about 2 h (Nakajima and Yokoi, 2005), and, with a 2 h half-life, plasma nicotine levels will accrue (e.g., 3 or more half-lives) with regular smoking during waking hours (Hukkanen et al., 2005); therefore, nicotine withdrawal likely did not confound any of our findings (for review see Sacco et al., 2004). Taken together, acute nicotine administration in smoking AUD may facilitate performance on some aspects of neurocognition; however, it appears

that increasing smoking intensity and/or chronicity in AUD is robustly related to poorer performance on multiple neurocognitive functions and may mitigate any enhancing effects of acute nicotine consumption, particularly with greater levels of smoking severity and/or chronicity. For further discussion of potential mechanisms associated with the neurocognitive and neurobiological effects of chronic cigarette smoking in AUD and non-clinical samples (see Durazzo and Meyerhoff, 2007; Durazzo et al., 2010b).

The most consistent finding for COMT in this alcohol dependent cohort was that Met allele carriers performed better than Val homozygotes on measures of executive skills and general intelligence. Specifically, Met homozygotes and Val/Met heterozygotes performed significantly better than Val homozygotes on the executive skills and general intelligence domains, respectively. Met homozygotes showed trends for better performance than Val homozygotes on the general intelligence and visuospatial skills domains. Moderate ES were apparent for the differences between COMT Met carriers and Val homozygotes. There were no significant differences between COMT Met homozygotes and Val/Met heterozygotes, and Val homozygotes were not superior to Met homozygotes on any neurocognitive domain. Our COMT findings for the executive skills domain in this alcohol dependent cohort are consistent with studies of the COMT rs4680 SNP in normal controls and individuals with neuropsychiatric disorders, which reported that Met homozygotes were superior to Val homozygotes on measures of executive skills (Savitz et al., 2006; Wishart et al., 2011). With respect to specific measures of executive skills, studies have found Met homozygotes made significantly less perseverative responses or perseverative errors on the WCST than Val homozygotes across cohorts of normal controls, individuals at risk for schizophrenia, and schizophrenics (Joober et al., 2002; Malhotra et al., 2002; Mattay et al., 2003; Rosa et al., 2004). COMT Met homozygotes in this report also made less perseverative errors and perseverative responses on the WCST than Val homozygotes ($p < 0.05$), after controlling for BDNF genotype, smoking status, lifetime alcohol consumption, age, and AMNART (data for individual tests not shown). The influence of the COMT Val158Met SNP on neurocognition may be related to its effects on the regulation of tonic and phasic dopamine activity (DA) in the frontal lobe neocortex. The G → A missense mutation in this SNP translates into a substitution of Val by Met at codon 158. Physiologically, the Val158Met SNP affects the thermostability of the COMT enzyme in a Met dose-dependent fashion such that Met homozygotes demonstrate approximately 50% reduction in enzymatic activity in the frontal lobe cortex (see Dickinson and Elvevag, 2009). The decreased enzymatic activity of COMT Met allele carriers is thought to result in higher tonic and more stable DA concentrations at paralimbic and neocortical D1 receptors and lower phasic alterations in subcortical DA levels, which is suggested to relate to better and more consistent performance on abilities subserved by the anterior frontal-subcortical circuits, particularly executive skills and working memory (see Bilder et al., 2004; Dickinson and Elvevag, 2009). The superior performance of Met carriers relative to Val homozygotes on measures of executive and intellectual skills is consistent with the suggested effects of COMT genotype on tonic-phasic DA neurotransmission in anterior frontal-subcortical circuits subserving higher order neurocognitive functions. Contrary to previous studies, the BDNF Val66Met polymorphism was not a significant predictor of any neurocognitive domain. ES for pairwise comparisons of BDNF genotypes across the 11 domains were trivial (0.01–0.15), which suggests the lack of significant findings in Val homozygotes and Met Carriers were not a function of insufficient statistical power.

Age was a significant predictor of all domains except of auditory-verbal learning and memory and working memory, *despite the use of age-adjusted norms*. Fast, flexible, and accurate responses are required for better scores on the predominantly non-verbal/visuospatial tasks comprising the cognitive efficiency, processing speed, and visuospatial skills domains, as well as on WAIS-III non-verbal tasks contributing to the general intelligence domain. Research on normal age-related changes in neurocognition suggests decreasing information processing speed is significantly related to the declines in learning, memory, and visuospatial abilities with increasing age (Salthouse, 1996, 2000; Christensen, 2001; Finkel et al., 2007; Kochunov et al., 2010). Overall, the age effects observed in this study are congruent with the "premature aging" hypothesis in AUD (Oscar-Berman, 2000). It is also noteworthy that, in this report, and in our earlier work (Durazzo et al., 2007c, 2008, 2010a) measures of alcohol consumption were not associated with neurocognition. This is consistent with other research that found measures of alcohol consumption quantity/frequency were weakly or not related to neurocognition (Schafer et al., 1991; Beatty et al., 1995, 2000; Eckardt et al., 1998; Horner et al., 1999; Sullivan et al., 2000).

This study has limitations that may influence the generalizability of the findings. The sample size of this study was modest, which did not permit a full factorial examination of all predictors (e.g., gene × gene interactions) and possibly led to inadequate power to detect other potential relationships between COMT and the neurocognitive domains investigated. We did not assess for personality disorders, which may contribute to the neurocognitive and neurobiological abnormalities observed in AUD (Eckardt et al., 1995; Kuruoglu et al., 1996; Giancola and Moss, 1998; Costa et al., 2000). Results may have also been influenced by factors not directly assessed in this study, such as diet, exercise, and exposure to environmental cigarette smoke or other premorbid/genetic variables. Finally, the majority of participants were males recruited from the San Francisco VA Medical Center, which did not allow for the examination of the potential effects of sex on neurocognition.

In summary, chronic cigarette smoking was strongly related to poorer performance on multiple neurocognitive domains, while the COMT Val158Met polymorphism showed significant associations with three domains (executive skills, general intelligence, and auditory-verbal memory) in this cohort of short-term abstinent alcohol dependent individuals. Importantly, our results indicate that the inferior performance demonstrated by smoking compared to non-smoking ALC was not mediated by the SNPs investigated, alcohol consumption, or comorbid medical and psychiatric conditions. The current findings reinforce our previous work that indicates consideration of smoking status and other prevalent comorbid conditions in AUD is critical to fully appreciate how this clinical syndrome influences neurocognition. Our results for the relationships of COMT polymorphism to neurocognition in AUD were consistent with findings in normal controls and individuals

with schizophrenia-spectrum disorders. Research investigating the influence of BDNF and COMT on neurocognitive recovery during sustained abstinence from alcohol in this cohort is clearly indicated. Cigarette smoking is a modifiable health risk that is directly associated with at least 440,000 deaths in the United States alone and 10 million annual deaths worldwide, with greater mortality and morbidity among those with substance use disorders, mood disorders, and schizophrenia (see Durazzo and Meyerhoff, 2007 for review). This study provides clinicians with additional information on the adverse consequences of chronic smoking in those seeking treatment for AUD. In the face of high mortality from cigarette smoking in AUD (Hurt et al., 1996), the data from this report in conjunction with other neurocognitive and neuroimaging studies (see Durazzo and Meyerhoff, 2007; Durazzo et al., 2010b), lend strong support to the expanding clinical movement (which is standard practice at the San Francisco VA Medical Center) to make smoking cessation programs available to smokers at the inception of treatment for alcohol/substance use disorders.

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Rapid effect of nicotine intake on neuroplasticity in non-smoking humans

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In various studies nicotine has shown to alter cognitive functions in non-smoking subjects. The physiological basis for these effects might be nicotine-generated modulation of cortical structure, excitability, and activity, as mainly described in animal experiments. In accordance, a recently conducted study demonstrated that application of nicotine for hours via nicotine patch in non-smoking humans alters the effects of neuroplasticity-inducing non-invasive brain stimulation techniques on cortical excitability. Specifically, nicotine abolished inhibitory plasticity independent from the focality of the stimulation protocol. While nicotine prevented also the establishment of non-focal facilitatory plasticity, focal synapse-specific facilitatory plasticity was enhanced. These results agree with a focusing effect of prolonged nicotine application on facilitatory plasticity. However, since nicotine induces rapid adaption processes of its receptors, this scenario might differ from the effect of nicotine in cigarette smoking. Thus in this study we aimed to gain further insight in the mechanism of nicotine on plasticity by exploring the effect of nicotine spray on non-focal and focal plasticity-inducing protocols in non-smoking subjects, a fast-acting agent better comparable to cigarette smoking. Focal, synapse-specific plasticity was induced by paired associative stimulation (PAS), while non-focal plasticity was elicited by transcranial direct current stimulation (tDCS). Forty eight non-smokers received nicotine spray respectively placebo combined with one of the following protocols (anodal tDCS, cathodal tDCS, PAS-25, and PAS-10). Corticospinal excitability was monitored via motor-evoked potentials elicited by transcranial magnetic stimulation (TMS). Nicotine spray abolished facilitatory plasticity irrespective of focality and PAS-10-induced excitability diminution, while tDCS-derived excitability reduction was delayed and weakened. Nicotine spray had thus a clear effect on neuroplasticity in non-smoking subjects. However, the effects of nicotine spray differ clearly from those of prolonged nicotine application, which might be due to missing adaptive nicotinic receptor alterations. These results enhance our knowledge about the dynamic impact of nicotine on plasticity, which might be related to its heterogenous effect on cognition.

Keywords: neuroplasticity, nicotine, non-smokers, PAS, tDCS

INTRODUCTION

Nicotine binds to the nicotinergic type of cholinergic receptors, which are ligand-gated cation channels. Nicotine also affects other transmitter systems by regulating the release of dopamine, adrenaline, serotonin, and glutamate, amongst others. Hereby nicotine is thought to be critically involved in the induction and modulation of neuroplasticity (Burnashev, 1998; Dajas-Bailador and Wonnacott, 2004; Levin et al., 2006), the likely physiological basis of learning and memory formation (Rioult-Pedotti et al., 1998, 2000). Specifically, in animal experiments the activation of nicotinic receptors results in a facilitation of long-term potentation (LTP), both dependent and independent of NMDA-receptor activation (Sawada et al., 1994; Huerta and Lisman, 1995; Matsuyama et al., 2000).

COGNITIVE EFFECTS OF NICOTINE

On a functional basis, in animal experiments nicotine has been shown to improve working memory function (Levin et al., 1994) and attention (Hahn and Stolerman, 2002), while in humans cognitive results are heterogeneous. Kleykamp et al. (2005) have found no effect of nicotine gum in different doses on attention and working memory in never-smokers. In contrast, other studies have shown that nicotine improves alerting attention-accuracy (Barr et al., 2008), visuospatial attention (Thiel et al., 2005), and working memory (Kumari et al., 2003) in non-smoking subjects. However, nicotinic plasticity modulation has been explored only in few studies in humans so far.

NICOTINIC PLASTICITY MODULATION

Recently it was shown that global cholinergic activation via the cholinesterase-inhibitor rivastigmine enhances focal plasticity induced by paired associative stimulation (PAS) and abolishes/reverses non-focal facilitatory plasticity generated by transcranial direct current stimulation (tDCS), thus resulting in a focusing effect of acetylcholine on facilitatory plasticity (Kuo et al., 2007). Similar results have been found for nicotinic effects

in non-smokers (Thirugnanasambandam et al., 2011). However, in difference to global cholinergic activation, nicotine reduced, or abolished inhibitory plasticity in these subjects. In principal accordance, nicotine enhanced the facilitatory effects of intermittent theta burst stimulation in the human motor cortex (Swayne et al., 2009). However, nicotine pharmacokinetics in the above-mentioned study of Thirugnanasambandam et al. (2011) differ from that present in cigarette smokers, because in that study nicotine patches were applied, which are characterized by a slow build-up and long duration of enhanced nicotine concentration, whereas cigarette smoking results in a fast build-up and decay of nicotine concentration. Since nicotinic receptor activation induces rapid adaptive processes like desensitization (Alkondon et al., 2000; Mansvelder and McGehee, 2000), upregulation, and greater density of AChR (Flores et al., 1992; Mukhin et al., 2008) these might have affected the impact of nicotine on plasticity relevantly in that study.

PLASTICITY INDUCTION PROTOCOLS

In the present study, we therefore aimed to mimic the pharmacokinetics of cigarette smoking by exploring the impact of nicotine spray, which results in maximal nicotine plasma concentration within a few minutes, on focal and non-focal plasticity. Facilitatory and inhibitory plasticity were induced by tDCS (Nitsche and Paulus, 2000, 2001) and PAS (Stefan et al., 2000). Both stimulation protocols induce non-invasively NMDA- and calcium channel-dependent plasticity (Stefan et al., 2002; Nitsche et al., 2003, 2004), though tDCS is supposed to induce non-focal plasticity changes due to affecting large neuron populations under large electrodes (Purpura and McMurtry, 1965; Nitsche et al., 2007), while PAS-induced plasticity is restricted to synaptic connections between somatosensory and motor cortex (Weise et al., 2006). As outlined above, in non-smokers nicotine patch abolished inhibitory plasticity and focused facilitatory plasticity. Since nicotine receptors are rapidly modified by chronic nicotine exposure, we hypothesize that administration of a single dose of fast-acting nicotine might affect plasticity differently as compared to nicotine patch.

MATERIALS AND METHODS

SUBJECTS

Altogether 48 otherwise healthy non-smoking subjects participated in this study. **Table 1** displays the characteristics of the subjects in terms of age and gender. All subjects were of Caucasian origin. Chronic and acute medical diseases or any history of neurological/psychiatric disease were excluded before entering the study by assessment of medical history, likewise intake of chronic and acute medication. Pregnancy, family history of epilepsy, presence of any metallic implant, or cardiac pacemaker were ruled out. All subjects gave written informed consent before participating in the study. The experiments were approved by the local Ethics Committee and conformed to the principles laid down in the Declaration of Helsinki. Allocation of the subjects to the respective experimental conditions as well as order of sessions was randomized.

PAIRED ASSOCIATIVE STIMULATION

Altogether twenty-four subjects participated in the PAS experiment. Twelve non-smokers participated in the inhibitory PAS protocol (PAS-10) and 12 in the excitatory PAS protocol (PAS-25). Peripheral nerve stimulation was delivered to the right ulnar nerve at the wrist level by a Digitimer D185 multipulse stimulator (Digitimer, Welwyn Garden City, UK) at an intensity of 300% of the sensory perceptual threshold followed by single pulse transcranial magnetic stimulation (TMS) applied with a stimulator output resulting in motor-evoked potentials (MEPs) of approximately 1 mV amplitude ("baseline intensity," see description in Section "Monitoring of Cortical Excitability"). The paired pulses were repeated 90 times at a frequency of 0.05 Hz. This protocol induces long-lasting excitability changes in the motor cortex depending on the interstimulus interval (ISI). An ISI of 10 ms induces excitability diminution (PAS-10) whereas an ISI of 25 ms induces facilitation (PAS-25; Stefan et al., 2000; Wolters et al., 2003). The PAS-protocols were combined with either nicotine or placebo spray for each subject in different experimental sessions.

Table 1 | Characteristics of the subjects participating in the experiments.

Stimulation Parameter	Anodal tDCS	Cathodal tDCS	PAS-10	PAS-25
Number of subjects	12	12	12	12
Number of females(%)	6(50)	7(58)	6(50)	6(50)
Age	24.4 ± 1.2	26.9 ± 3.6	25.9 ± 2.1	24.5 ± 1.3
S1 mV before nicotine spray	41.3	45.6	43.9	43.6
S1 mV after nicotine spray	42.3	44.9	44.4	43.3

TRANSCRANIAL DIRECT CURRENT STIMULATION

Twenty four subjects participated in the tDCS experiments (12 non-smoking subjects participated in the inhibitory tDCS protocol and 12 in the excitatory tDCS protocol). We used a battery-driven constant current stimulator (Schneider Electronics, Gleichen, Germany) with a maximum output of 2 mA. tDCS was administered via rubber electrodes covered by saline soaked sponges (35 cm^2). One electrode was positioned over the motor cortex representational area of the right abductor digiti minimi muscle (ADM), the other electrode above the right orbit. All subjects received 1 mA of either anodal or cathodal stimulation for 13 min (anodal tDCS) or 9 min (cathodal tDCS), which had been demonstrated to induce cortical excitability enhancement or inhibition lasting for about 1 h after the end of stimulation (Nitsche and Paulus, 2001; Nitsche et al., 2003) combined with nicotine spray or placebo medication in different experimental sessions.

Monitoring of motor cortex excitability

Transcranial magnetic stimulation-elicited MEPs were recorded to measure excitability changes of the representational motor cortical area of the right ADM. Single pulse TMS was conducted by a Magstim 200 magnetic stimulator (Magstim Company, Whitland, Dyfed, UK) at a frequency of 0.25 Hz with a figure of eight-shaped coil (diameter of one winding 70 mm, peak magnetic field, 2.2 T). The coil was held tangentially to the scalp at an angle of 45° to the sagittal plane with the coil handle pointing laterally and posterior. The optimal position was defined as the site where

stimulation resulted consistently in the largest MEPs. Surface EMG was recorded from the right ADM with Ag–AgCl electrodes in a belly tendon montage. The signals were amplified and filtered with a time constant of 10 ms and a low-pass filter of 2.5 kHz, then digitized at an analog-to-digital rate of 5 kHz and further relayed into a laboratory computer using the Signal software and CED 1401 hardware (Cambridge Electronic Design). The intensity was adjusted to elicit, on average, baseline MEPs of 1 mV peak-to-peak amplitude, and was kept constant for the post-stimulation.

PHARMACOLOGICAL INTERVENTION

Each subject participated in two sessions in randomized order. Nasal spray contained nicotine or inactive placebo. Nicotine was administered by nasal spray, containing 10 mg/ml nicotine, in a cumulative dose of 1 mg (Nicorette ®Nasal Spray, McNeil Products, UK) to all subjects in combination with one of the stimulation protocols, anodal tDCS, cathodal tDCS, PAS-10, and PAS-25. The rise time of nicotine by nasal spray in venous blood levels is close to venous blood levels of nicotine delivered by cigarets (Schneider et al., 1995) with a plasma peak level after 5–10 min. Side effects of the nasal nicotinic administration were coughing, sneezing, throat irritation, and dizziness like it has been described from prior clinical trials (Sutherland et al., 1992). Symptoms subsided rapidly after some minutes. To have a comparability to the nicotine patch study, we chose a dose of nicotine spray (1 mg) that delivers nicotine blood levels comparable to those of nicotine patch (8–9 ng/ml, see also Tønnesen et al., 1991; Pomerleau et al., 1992).

COURSE OF THE EXPERIMENT

Subjects were seated comfortably in a reclined chair with head- and armrest and asked to relax completely. EMG electrodes were placed at the right ADM as described above. Their exact position was marked with a pen. Then TMS was applied over the left representational area of the right ADM to determine the spot with the consistently highest MEPs in the resting ADM (optimal site), which was then marked with a pen. The TMS-intensity was adjusted to elicit MEP amplitudes of 1 mV (S1 mV). Twenty MEPs were recorded at this stimulus intensity and the mean MEP amplitude was calculated at baseline. Then nicotine nasal spray respective placebo spray was administered. Side effects like coughing and sneezing, that could interfere with the measurements subsided quickly and 10 min later one of the stimulation protocols, either tDCS or PAS, was administered, followed by immediate recording of at least 20 MEPs at the time points of 0, 5, 10, 15, 20, 25, 30, 60, 90, and 120 min. For the nicotine spray condition, the after-measurements were also conducted the evening of the stimulation day and in the morning and evening of the day following the plasticity induction procedure. Sessions were conducted in randomized order, and an inter-session interval of at least 1 week was obligatory to avoid interferences. See also **Figure 1** for course of the experiment.

DATA ANALYSIS AND STATISTICS

For all subjects the means of 20 MEP amplitudes recorded at each time point was calculated. The post-intervention mean MEP amplitudes from each subject were then normalized to the respective individual mean baseline MEP amplitude (quotient of post- vs. pre-intervention MEP amplitudes). Statistical analysis used SPSS general linear model analysis for variances for repeated measurements on normalized data. MEP amplitude was the dependent variable including all time points up to 120 min after stimulation. Drug (nicotine vs. plc) and time points were included as within-subjects factors. Stimulation protocol (atDCS, ctDCS,

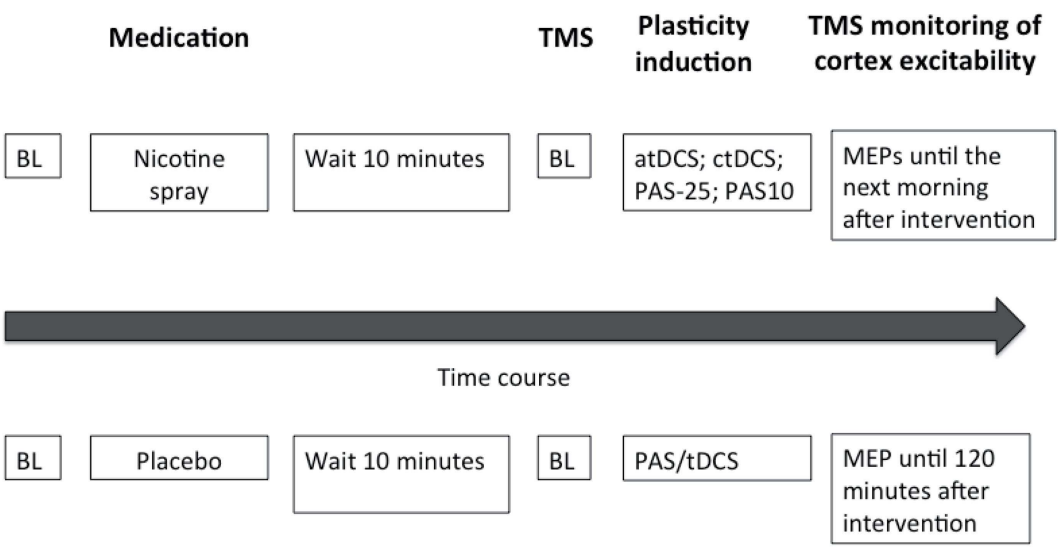

FIGURE 1 | This figure illustrates the experimental protocol of our study. At the beginning of the session baseline measurements (BL) were performed and followed by administration of either nicotine spray or placebo spray. After 10 min baseline (BL) motor cortical excitability was redetermined via TMS-induced motor-evoked potentials (MEP). One of the four excitability-inducing protocols were then applied (atDCS, ctDCS, PAS-10, PAS-25). After-measurements started immediately after the application of the protocols, and were conducted every 5 min for the first 30 min, then every 30 min up to 120 min for both sessions. MEP amplitudes were also recorded the next morning and next evening for the nicotine spray sessions.

PAS-25, and PAS-10) served as between-subject factors. Mauchly's sphericity test was performed and Greenhouse–Geisser correction was applied when necessary. Student's t-tests (paired samples, two-tailed, $p < 0.05$, not adjusted for multiple comparisons) were performed to compare the MEP amplitudes before and after the interventional brain stimulations in each condition and between drug conditions (nicotine/placebo) for each time point. A p-value of <0.05 was considered significant for all statistical analyses. Significances of differences in demographic factors were tested by one-way ANOVA and chi-square for gender. All data are expressed as mean ± standard of error (SEM).

RESULTS

All subjects tolerated the experiments well, even though nearly all of the subjects (44 of 46 subjects, 96%) complaint about sneezing and coughing after inhalation of nicotine spray. No significant group differences were found in terms of age, gender, TMS-intensity to elicit an MEP of 1 mV (S1 mV) before and after administration of nicotine spray (see **Table 1**). Absolute baseline MEP amplitudes did not differ significantly within and between stimulation groups and medication conditions (Student's t-test, two-tailed, unpaired/paired, $p > 0.05$ for all cases). The ANOVA revealed a significant main effect of the between-subject factor timepoint and stimulation. The interactions drug × stimulation, timepoint × stimulation, and drug × timepoint × stimulation were also significant (see also **Table 2** for results of degrees of freedom, F-value, p-value, *indicates significant values with $p < 0.05$).

EFFECTS OF NICOTINE SPRAY ON tDCS-INDUCED PLASTICITY IN NON-SMOKERS

In the PLC condition the anodal tDCS-induced excitability increased MEP amplitudes stayed significant until 90 min after stimulation, and the cathodal tDCS-induced inhibition lasted until 90 min after tDCS. As revealed by the *post hoc* t-test (paired, two-tailed, $p < 0.05$) nicotine spray abolished the atDCS-induced long-lasting excitability enhancements in non-smoking subjects. For the cathodal tDCS protocol under influence of nicotine spray excitability diminuation started delayed after 15 min and lasted only until 20 min after stimulation. A second diminuation peak could be seen after 90 min and lasted until 120 min post-stimulation (**Figure 2**). Thus nicotine spray administration delayed and weakened the ctDCS-induced after-effects.

Table 2 | Results of the ANOVA.

Parameters	*df*	*F*-Value	*p*-value
Drug	1	2.288	0.138
Timepoint	6.932	2.163	0.038*
Stimulation	3	11.423	0.001*
Drug × stimulation	3	8.673	0.001*
Timepoint × stimulation	30	2.056	0.001*
Drug × timepoint	5.446	1.478	0.192
Drug × timepoint × stimulation	30	1.831	0.027*

*$p < 0.05$

EFFECTS OF NICOTINE SPRAY ON PAS-INDUCED PLASTICITY IN NON-SMOKERS

As revealed by Student's t-test (paired, two-tailed, $p < 0.05$) in the PLC- condition PAS-25-induced excitability changes (MEP-enhancements) were significantly increased for up to 90 min after stimulation, while the PAS-10-induced long-lasting after-effects returned to baseline 120 min after PAS. Nicotine spray abolished both the PAS-10-induced inhibitory MEP-changes and the PAS-25-induced excitatory after-effects completely (**Figure 3**). Thus nicotine spray prevented the induction of PAS-induced focal plasticity.

EFFECT OF NICOTINE SPRAY ON MOTOR CORTEX EXCITABILITY MEASURED BY TMS-ELICITED MOTOR-EVOKED POTENTIALS

To rule out, that nicotine spray itself increases or decreases motor cortex excitability measured by TMS, we compared TMS-intensity needed to elicit an MEP of 1 mV before and after the administration of nicotine spray. The respective Student's t-test (paired, two-tailed) did not reveal any significant difference between different stimulation groups and before vs. after nicotine (see **Figure 4**).

DISCUSSION

The results of the present study show that nicotine administration has a prominent and rapidly evolving effect on stimulation-induced neuroplasticity in the human primary motor cortex. In non-smoking humans, nicotine spray prevented completely the induction of focal and non-focal plasticity, as induced by PAS-25, and anodal tDCS. Moreover it reduced excitability-diminishing plasticity accomplished by PAS-10, whereas nicotine had no major impact on cathodal tDCS-generated plasticity.

These results are only in partial accordance with those of a former study in our group, where we explored the effects of long-acting nicotine, administered via patch, on plasticity induced by the same stimulation techniques (Thirugnanasambandam et al., 2011). Here nicotine abolished inhibitory plasticity regardless of its focality, whereas facilitatory plasticity was enhanced, when the induction procedure was focal, but abolished, when it was non-focal. Moreover, the abolishing effect of nicotine spray on facilitatory plasticity in the present study differs prominently from the effects of nicotine lozenge application, which enhanced facilitatory plasticity induced by intermittent theta burst stimulation of the human motor cortex (Swayne et al., 2009). The comparison between the present study and that of Thirugnanasambandam, in which resulting nicotine plasma level should be more or less identical, is in favor for an impact of the duration of nicotine application on human cortical plasticity. One reason for these different effects of acute (in terms of minutes), and chronic (in terms of hours) nicotine administration on plasticity might be adaptive receptor up- or downregulation, which takes place rapidly in nicotinic receptors (Flores et al., 1992; Alkondon et al., 2000; Mukhin et al., 2008; Mansvelder and McGehee 2000) and thus might have had an impact on the results of the patch study. With regard to the study of Swayne and co-workers, some other aspects differ between the respective studies, such as kind of stimulation protocol, duration of plasticity induced by the stimulation protocol alone, expected plasma level, and pharmacokinetics, which makes a comparison between studies difficult, but nevertheless is

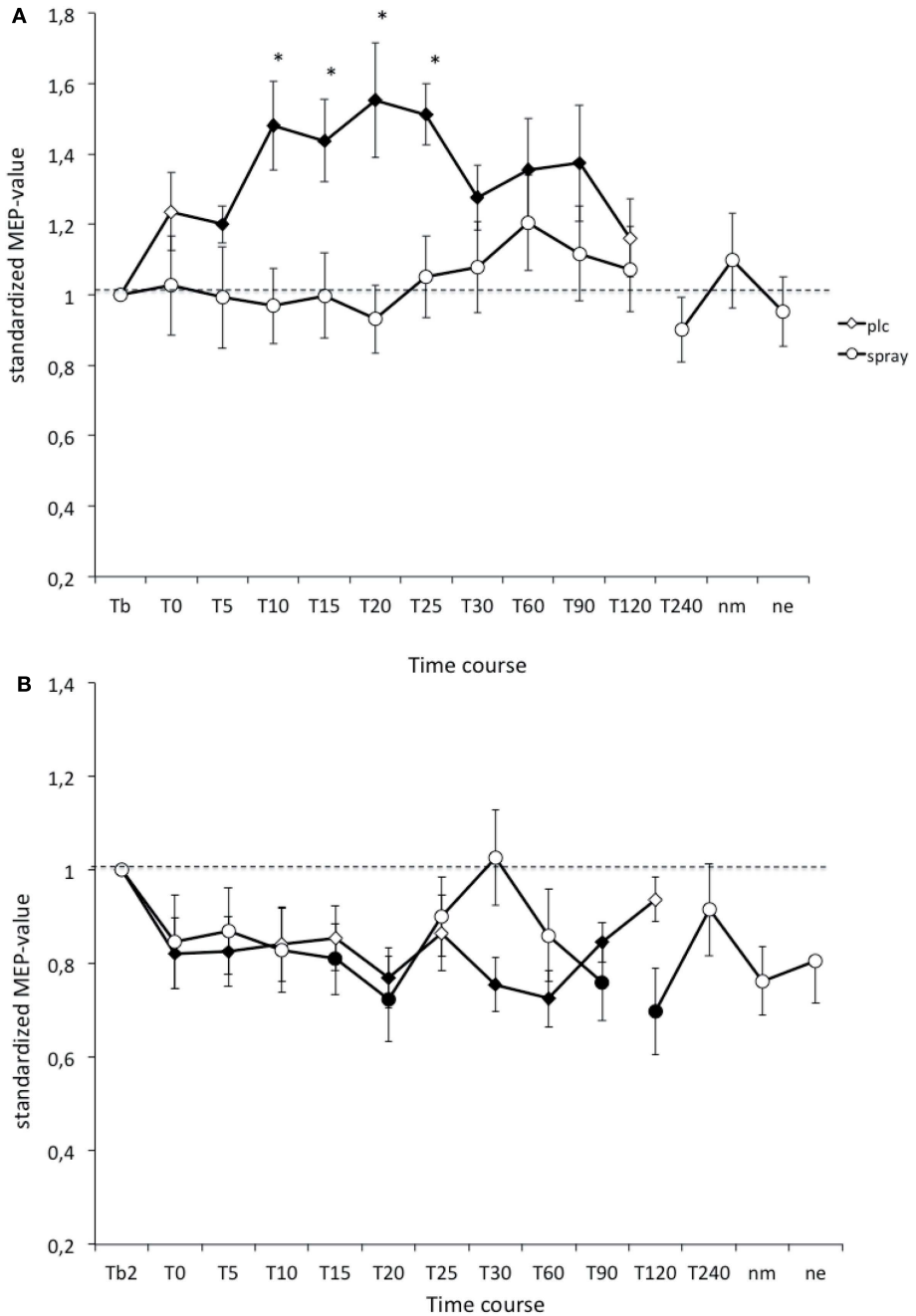

FIGURE 2 | (A,B) Nicotinergic impact on transcranial direct current stimulation (tDCS) induced neuroplasticity. Shown are the graphs with motor evoked potentials (MEP) standardized to the baseline on the *Y*-axis plotted against different time points post-intervention on the *X*-axis. Filled symbols indicate statistically significant deviations from baseline and asterisks indicate significant differences between the control and nicotine conditions (Student's *t*-test, paired, two-tailed, $p < 0.05$). nm, Next morning; ne, next evening; plc, placebo. Tb, Baseline MEP-amplitude before begin of the stimulation protocols (standardized). Error bars indicate standard error of mean.

in advance for a neuromodulatory effect of nicotine on plasticity, whose direction might be determined by diverse factors.

PROPOSED MECHANISM OF ACTION

The results of the present study allow no definite conclusions about how nicotine has affected plasticity in the present study, but some candidate mechanisms can be derived from the more general functions of nicotinic receptors, and the physiological basis of tDCS, and PAS. Nicotine binds to nicotinergic ACh-receptors that are widely distributed throughout the brain (Albuquerque et al., 2009). In the brain two major subunits compositions exist, the heteromeric assembly of α4β2

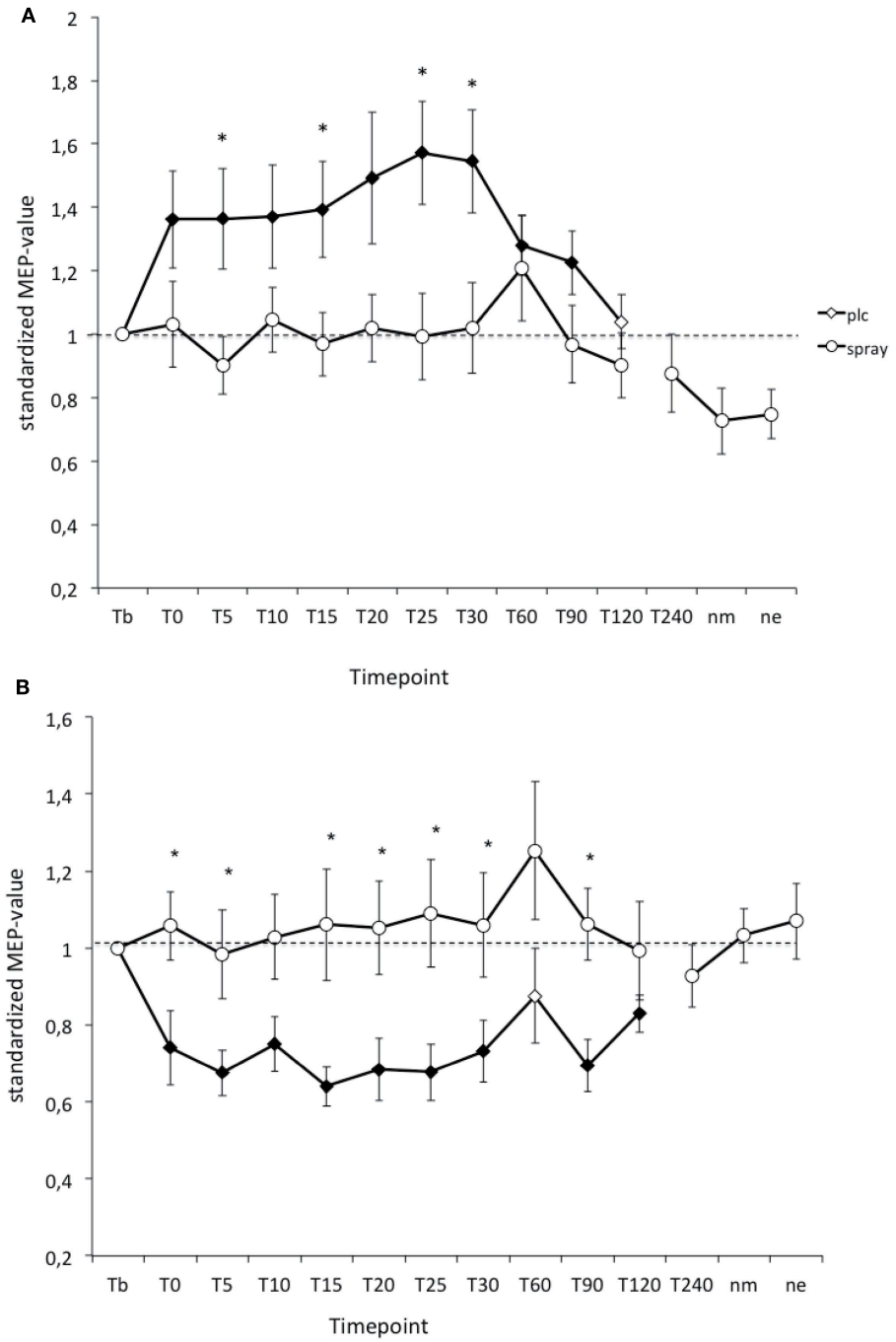

FIGURE 3 | (A,B) Nicotinergic impact on paired associative stimulation (PAS) induced neuroplasticity. Shown are the graphs with motor evoked potentials (MEP) standardized to the baseline on the *Y*-axis plotted against different time points post-stimulation on the *X*-axis. Filled symbols indicate statistically significant deviations from baseline and asterisks indicate significant differences between the placebo medication and nicotine conditions (Student's *t*-test, paired, two-tailed, $p < 0.05$). nm, Next morning; ne, next evening; plc, placebo. Tb, Baseline MEP-amplitude before begin of the stimulation protocols (standardized). Error bars indicate standard error of mean.

and the homomeric α7 subunit, both exhibiting different pharmacological and physiological properties (Jones et al., 1999), and both increasing intracellular calcium levels by serving as pre- and postsynaptic ligand-gated calcium channels. As the after-effects of tDCS and PAS are likewise calcium-dependent (Stefan et al., 2002; Nitsche et al., 2003), a possible effect of nicotine on neuroplasticity might be alteration of intracellular calcium levels. The amount of intracellular calcium determines if inhibitory, facilitatory, or no plasticity is induced (Lisman, 2001; Misonou et al., 2004). Given that nicotine enhances intracellular

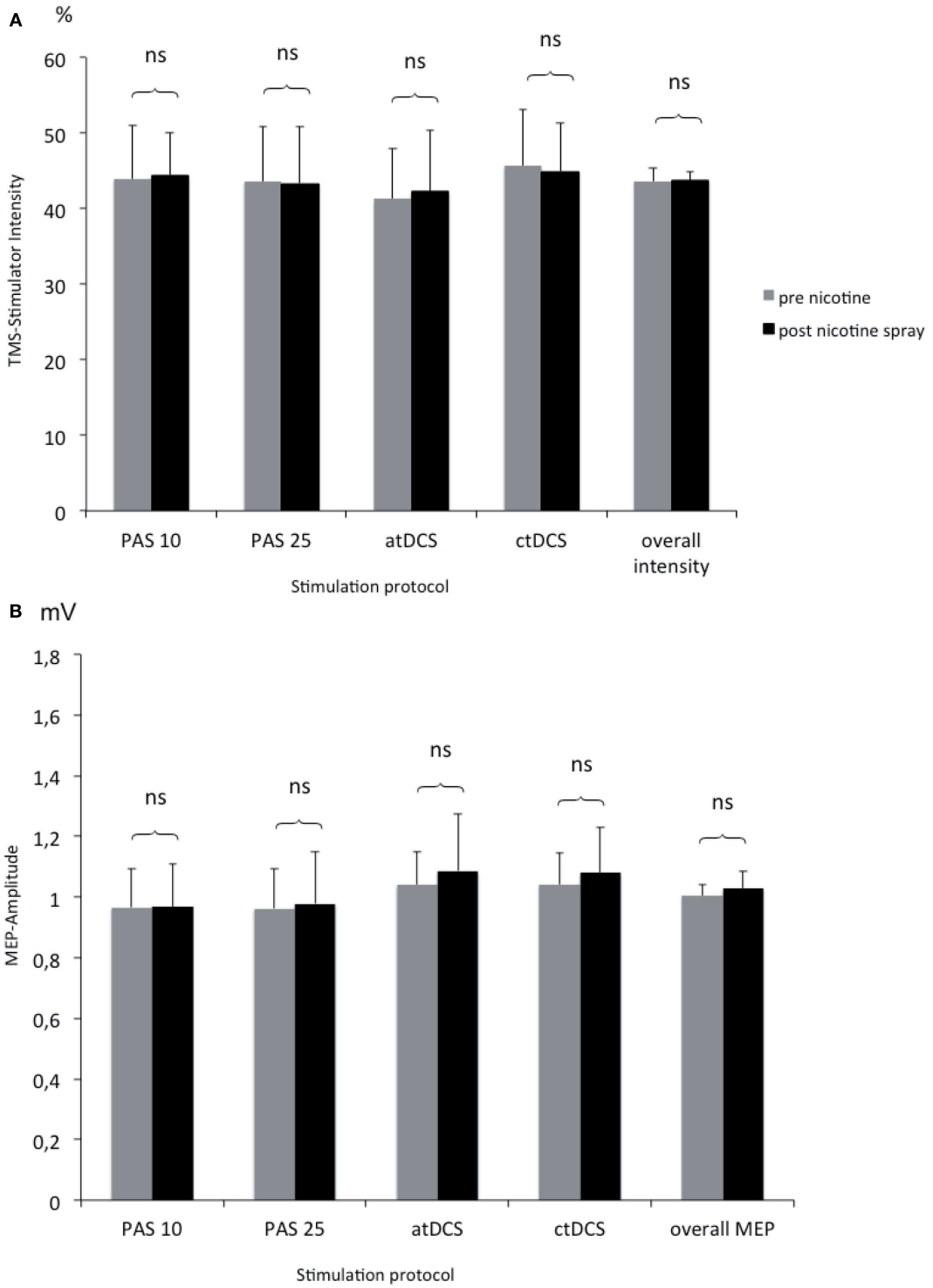

FIGURE 4 | (A,B) Comparison of TMS-stimulator intensity and MEP amplitudes before and after nicotine spray administration. Shown are the TMS-stimulator intensity as percentage of maximum stimulator output **(A)** and respective motor evoked potentials [MEP amplitudes in mV; **(B)**] before and after nicotine spray administration for the different interventions (atDCS, ctDCS, PAS-25, PAS-10). ns, Non significant; mV, millivolt.

calcium concentration via activation of the respective nicotinic receptors, nicotine administration might have caused a calcium concentration that overshoots the limit for LTP-like induction elicited by atDCS and PAS-25, thus resulting in an extinction of after-effects. The same might be true for LTD-induction processes.

This proposed mechanism of action is however hypothetical and highly speculative presently and needs to be further explored experimentally in the future. Due to the complex impact of nicotine on other neuromodulators and – transmitters, alternative mechanisms of action cannot be ruled out.

GENERAL REMARKS

The results of our study demonstrate that nicotine spray influences neuroplasticity in non-smoking humans prominently. Nicotine spray in abolishes/reduces focal and non-focal plasticity. Here the connection to cognitive studies is not easily drawn, since the nicotinic effect on cognition and memory in non-smokers is discussed controversially. A review of Heishman (1998) reported about no true enhancement of sensory ability, selective attention, learning, and other cognitive abilities (e.g., problem solving, reasoning) in non-smoking subjects. Ernst et al. (2001) describe a nicotinic improvement of reaction time, but no effect on working memory in non-smokers. Poltavski and Petros (2005) have even shown a decrement in working memory in non-smokers. To further complicate argumentation the majority of the studies have used nicotine devices other than nicotine spray (nicotine patch, cigarets, inhaler), thus comparability to these studies is difficult because of different bioavailability. Only few studies have used nicotine spray in non-smokers and have found amelioration in rapid visual information processing (Rusted and Alvares, 2008) and fine motor-skills (Perkins et al., 2008). Potential connections of nicotinergic changes in LTP- and LTP-like plasticity and alterations in cognitive functions in smokers and non-smokers have to be explored more directly and intensively in the future. To clarify the specific receptor mechanisms, different nicotinic receptor subtypes have to be examined by pharmacological interventions (agonists and antagonists). Moreover further exploration of genetic differences between smokers and non-smokers might give further insight into the question, why people develop a nicotine addiction.

LIMITING CONDITIONS

Some limitations of the present study should be taken into account. The study was conducted in a single-blinded manner, so that the person carrying out the experiments knew about the condition of the subject (nicotine spray vs. placebo), thus delivering a possible confounding factor. Furthermore, TMS-measurements of cortical excitability were not performed until the next day in the placebo group, which complicates evaluation of nicotinic long-lasting effects on plasticity. Another limitation is the fact, that we cannot exclude non-linear dose-dependent effects of nicotine spray, since we performed the study with a stable dose of 1 mg. Dose-related effects on cortical excitability have p.e. been described for dopamine (Monte-Silva et al., 2009) and possible have to be taken into account also for other substances. This might be of special importance for the physiological effects of neuromodulators. Another restriction is that we did not obtain blood levels of nicotine, thus it cannot be excluded completely that different effects of nicotine patch and spray applications on plasticity are at least partially caused by different blood concentrations. It might furthermore complicate the comparability of the results of this study with other studies as well as cognitive testing. Still former pharmacological studies have measured blood levels in subjects taking 1 mg nicotine spray. With an average of 8–9 ng/ml, the blood level did not differ from the blood levels obtained after administration of nicotine patch 15 mg/16 h (8,9 ng/ml), which is the dosage we had chosen for the patch study (Tønnesen et al., 1991; Pomerleau et al., 1992; Thirugnanasambandam et al., 2011).

Moreover it should be mentioned that the explanation and discussion of the results is only hypothetical presently as currently direct correlation to cognitive studies is still missing. Possible future studies might test this linking between cortical excitability changes and impact on cognitive functions more directly and give further insight in the mechanisms of nicotine in smokers and non-smokers. Since chronic nicotine spray administration has fast and similar effects on craving and withdrawal symptoms than patch (Hajek et al., 1999) it would be further interesting in future possible studies to explore long-term effects of nicotine spray on neuroplasticity.

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The Yin and Yang of nicotine: harmful during development, beneficial in adult patient populations

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Nicotine has remarkably diverse effects on the brain. Being the main active compound in tobacco, nicotine can aversively affect brain development. However, it has the ability to act positively by restoring attentional capabilities in smokers. Here, we focus on nicotine exposure during the prenatal and adolescent developmental periods and specifically, we will review the long-lasting effects of nicotine on attention, both in humans and animal models. We discuss the reciprocal relation of the beneficial effects of nicotine, improving attention in smokers and in patients with neuropsychiatric diseases, such as schizophrenia and attention deficit/hyperactivity disorder, vs. nicotine-related attention deficits already caused during adolescence. Given the need for research on the mechanisms of nicotine's cognitive actions, we discuss some of the recent work performed in animals.

Keywords: nicotine, developmental stages, animal model, brain development, ADHD

INTRODUCTION

Smoking is the leading preventable cause of death and disability in the USA (Novick, 2000), and nicotine, the main active compound in tobacco smoke can have health effects in very different ways. Obviously, the best known is its highly addictive property. In addition, it has various more subtle effects on the brain. Two prominent features are its effect on brain developmental and on attention. Moreover, nicotine has been shown to exert a protective effect on the display of neurodegenerative diseases. The mechanisms behind the adverse and potentially beneficial effects will need more research in the years to come. This review will highlight some of the salient features of nicotine along these lines.

With respect to development, there are two main developmental periods during which individuals are at risk to come into contact with biologically relevant doses of nicotine. The first window of vulnerability is during prenatal development, when women might smoke during pregnancy. The second is the developmental period of adolescence, during which when most smokers start their habit (Chassin et al., 1996). In adulthood the effects of nicotine may be less on neuronal development, but become apparent in its acute effects on neuronal circuitry properties (for review Poorthuis et al., 2009). This has immediate consequences for the attention state of the brain (for review Counotte et al., 2011b). Related to this, smokers use nicotine to self-medicate their attention deficits (Lerman et al., 2001), and patients with attention deficit/hyperactivity disorder (ADHD) perform worse when abstinent from nicotine (McClernon et al., 2008). ADHD is a common clinically significant condition in school-aged children, affecting 5–10% of children worldwide, with persisting lifelong features (Pediatrics, 2000). There are reciprocal links between smoking and ADHD, with on one hand nicotine exposure during development increasing the risk for ADHD, and on the other hand high rates of ADHD patients that are smokers, probably (at least partly) to alleviate their attention deficits. Thus, despite negative effects of nicotine on brain development and its persistent addictive properties, there are instances in which nicotine exposure can be beneficial for an individual.

Another interesting feature of nicotine is its reported long-term benefit in protection to neurodegenerative disease (Bordia et al., 2008; Echeverria et al., 2010, for review, see Shimohama, 2009). Both nicotine and its breakdown product cotinine have been suggested as cognitive enhancers for Parkinson's and Alzheimer's disease in preclinical models (Bordia et al., 2008; Echeverria et al., 2010). Although potentially of interest, this aspect of nicotine action will not be discussed further here.

In this review, we will examine the long-term effects of nicotine exposure during two epochs of brain development (prenatal and peri-adolescent) in the attention domain of cognition. In humans, it is difficult to separate the effects of developmental nicotine exposure from confounding factors, such as demographics and pre-existing or co-morbid psychiatric disorders, which is why we will also review studies using animal models of developmental nicotine exposure. As recent studies have established a correlation of developmental nicotine use and attention, and studies describing the mechanisms behind these effects start to emerge, we will focus on studies assessing attention performance. Recent work using animal models has enabled us to study the molecular and synaptic mechanisms underlying the long-term effects of developmental nicotine exposure.

PRENATAL NICOTINE EXPOSURE

Even though smoking by pregnant women is declining in recent years, still 13% of women reported smoking during pregnancy in

2005 (Center for Disease Control and Prevention, 2009), which might even be an underestimation due to non-disclosure (Dietz et al., 2010). In some high-risk populations, smoking rates are as high as 25% (Arria et al., 2006). Many women find it hard to quit smoking when they are pregnant (Einarson and Riordan, 2009). Recommending nicotine replacement therapy may not be beneficial (Slotkin, 2008) considering that nicotine can cross the placenta and thus will enter the fetus through the mother's circulation. The developing fetus does not have the abilities to breakdown nicotine and its active metabolite cotinine as efficient as adults do, so nicotine and cotinine levels will buildup in the fetus (Sastry et al., 1998). A well-known more immediate consequence of maternal smoking during pregnancy is the increased risk for sudden infant death syndrome due to nicotine targeting monoamine pathways in brainstem and cardiac sympathetic innervation (Slotkin et al., 1999, 2010), and intra-uterine growth retardation resulting in reduced birth weight (Ernst et al., 2001b). Although it does not outweigh the negative effects of smoking, smoking during (late) pregnancy could protect the mother from hypertension and resulting pre-eclampsia (England and Zhang, 2007; Wikstrom et al., 2010).

PRENATAL NICOTINE EXPOSURE AND DEVELOPMENT OF ADHD

There is a substantial body of literature of both retrospective population-based studies and case-control studies suggesting that prenatal nicotine exposure is associated with an increased occurrence of ADHD (Milberger et al., 1996; Thapar et al., 2003; Schmitz et al., 2006; Biederman et al., 2009; Galera et al., 2011; Sagiv et al., 2012; for review Winzer-Serhan, 2008; Cornelius and Day, 2009). Milberger et al. (1996) found a 2.7-fold increase in ADHD associated with maternal smoking, when comparing 140 boys with ADHD to 120 control boys and their first-degree biological relatives. Galera et al. (2011) found that prenatal tobacco exposure has a risk factor of 1.41 for attention deficits and impulsivity in a longitudinal cohort of 2057 individuals who were followed from 5 months of age to 8 years. Already shortly after birth, infants exposed to tobacco smoking *in utero* showed poorer attention skills (Espy et al., 2011). In patients with ADHD, heavy maternal smoking is associated with poorer performance on the continuous performance task (CPT; Motlagh et al., 2011). However, a causal link between maternal smoking and ADHD has not been established. Using a different experimental design examining 815 families in which infants were divided into two groups, one group that was genetically related to their parents, and one that was genetically unrelated to their mothers because of fertility treatments that used donor eggs, Thapar et al. (2009) found that ADHD was only related to maternal smoking in the genetically related infants, even though confounding factors (like parental ADHD) were controlled for. In the genetically unrelated infants, maternal smoking did lead to a decreased birth weight, but was not associated with ADHD, suggesting that in traditional observational designs it is impossible to adequately control for confounding factors. This also suggests that the link between maternal smoking and ADHD might be more complicated, involving the interaction of genetic vulnerability and environmental influences including nicotine exposure.

PRENATAL NICOTINE AND GENE × ENVIRONMENT INTERACTIONS EXPLORED IN ANIMAL RESEARCH

A way to have better control over confounding factors and complex gene × environment interactions in humans is to use an animal model. Importantly, nicotine in rodents was found to have similar rewarding properties and cognitive effects to humans. This makes rodent nicotine research to large extent valid to in translating to human brain development, addictive properties, and attention.

Prenatal exposure to nicotine in rodents has been shown to modulate normal developmental activation of nAChRs, which is of importance for cell survival, synapse formation, and synapse maturation (for review Dwyer et al., 2009). Rats that had been previously exposed to nicotine *in utero*, in a paradigm where mothers were exposed to 0.06 mg/ml nicotine in the drinking water before and during pregnancy, show impairments in attention performance in the 5-CSRTT, both when tested during adolescence and adulthood (Schneider et al., 2011, 2012). Specifically, animals tested as adults showed decreased correct responses and an increased number of omissions (not paying attention; Schneider et al., 2011). Animals tested during adolescence only showed an increase in anticipatory responses but no difference in accuracy (Schneider et al., 2012). Also, prenatal nicotine exposure caused increased motor impulsivity in adult animals, indicated by the increased number of anticipatory responses, but it did not cause an increase in impulsive choice, since there was no difference in delay-discounting (Schneider et al., 2011). In addition, in a paradigm modeling third trimester nicotine exposure, where pups were exposed to 6 mg/kg nicotine per day by gastric intubation from P1 to P7, it was confirmed that this did not lead to differences in impulsive choice, and similarly, there were no differences in risky decision-making (Mitchell et al., 2012). Together, these findings show that prenatal nicotine exposure does lead to attention deficits, but animals show only some of the cognitive deficits (e.g., impulsive action) that are observed in humans. This suggests that some deficits may be due to human-specific genetic or environmental factors, or are due to other components than nicotine in tobacco smoke (Baker et al., 2004).

ADOLESCENT NICOTINE EXPOSURE

Brain development continues during adolescence, and nicotine from tobacco smoke can interfere with normal development (for review Slotkin et al., 2007; Counotte et al., 2011b), thus leading to deficits in attention and impulsivity. Jacobsen et al. (2005, 2007) showed that adolescent smokers perform worse on working memory and attention tasks. Individuals who were exposed to prenatal maternal smoking were even more severely impaired than those that were not (Jacobsen et al., 2007). Similarly, there were gender differences; female adolescent smokers were impaired on both a visual and auditory attention task, whereas male adolescent smokers were only impaired on the auditory attention task (Jacobsen et al., 2007). It is important to note that the smokers were allowed to smoke during a break between the tests to make sure they were not inattentive because of withdrawal from nicotine (West and Hack, 1991). These deficits in attention are accompanied by reduced attention-associated prefrontal cortical blood-oxygen level dependent (BOLD)-responses (Musso et al., 2007), indicating the importance of the prefrontal cortex in attention.

Smokers in general have been reported to have higher levels of impulsivity, both impulsive choice (Bickel et al., 1999; Mitchell, 1999) and impulsive action, or inhibitory control (Mitchell, 1999; Spinella, 2002; Skinner et al., 2004). However, it is difficult to determine whether nicotine exposure leads to impulsivity, or that impulsivity leads people to start smoking. There is currently no data showing that adolescent smokers have increased impulsivity or that adolescent nicotine exposure has long-lasting effects on impulsivity.

To address the issue of causality, we used an animal model to study the long-term effect of adolescent nicotine exposure on attention and impulsivity and found that 10 days of adolescent nicotine exposure (three daily injections of 0.4 mg/kg) impairs attention in the 5-CSRTT in adult animals, even after a relatively long nicotine-free period (Counotte et al., 2009, 2011a). Also, these animals showed increased motor impulsivity, indicated by an elevated number of premature responses in the 5-CSRTT but no deficits in impulsive choice (Counotte et al., 2009). We found that the decrease in attention following adolescent nicotine exposure was (at least in part) caused by a decreased synaptic expression of the metabotropic glutamate receptor mGluR2 in the medial prefrontal cortex, because stimulation of this receptor by local infusion of an mGluR2/3 agonist relieved the attention deficits and brought the adolescent nicotine exposed animals back to the level of their control counterparts (Counotte et al., 2011a). This change in mGluR2 signaling in the mPFC in turn leads to an alteration of the rules for spike timing-dependent plasticity, meaning that the ability to filter information has decreased (Gorioununova and Mansvelder, 2012). Thus, adolescent nicotine exposure affects synaptic signaling mechanisms involving metabotropic glutamate signaling in the mPFC. These signaling mechanisms are known to be important for plasticity and synaptic maturation (Michalon et al., 2012).

BENEFICIAL EFFECTS OF NICOTINE-LIKE SUBSTANCES

Nicotine has complex effects on cognitive performance that are in part determined by the existing state of the cholinergic system and by signaling via nicotinic receptors. Smokers, healthy non-smokers, and patients with impaired prefrontal cortical function, all differ in to what extent nicotine affects their cognitive performance. In healthy subjects, nicotine has no or only weak effects on cognitive performance. However, subjects with suboptimal performance, such as patients with ADHD, schizophrenia, or Alzheimer's disease are more likely to benefit from nicotine, and nicotinic drugs can act beneficial on attention and sensory gating (Newhouse et al., 2004). Compared with the percentage of smokers in the general population [currently around 20% in the US (Services and U. S. Department of Health and Human Services, 2010), a higher percentage of mentally ill patients smoke regularly (26–88%, depending on the mental illness; Lasser et al., 2000)]. Particularly patients with schizophrenia, depression, post-traumatic stress disorder, or ADHD smoke, and they have a lower chance of quitting smoking (Lambert and Hartsough, 1998; Services and U. S. Department of Health and Human Services, 2010). It has been postulated that tobacco smoking may ameliorate some of the major cognitive deficits in mentally ill patients and may act as self-medication (Lerman et al., 2001; Newhouse et al., 2004). On the other hand, abstinence worsens performance on attention tasks such as the human CPT in ADHD patients, but not in controls (McClernon et al., 2008). In healthy non-smokers the evidence of beneficial effects from nicotine on cognition is less clear. Performance in some cognitive tasks shows improvement by nicotine, whereas other aspects of cognition are impaired (Levin et al., 1997, 1998; Ernst et al., 2001a; Wignall and de Wit, 2011). For example, in non-smokers, nicotine can lead to faster reaction times in attention and working memory tasks, although this improvement might come at the expense of fewer correct responses (Le Houezec et al., 1994; Foulds et al., 1996). It is of note that even in smokers, the presence of nicotine may not necessarily improve their attention to the levels of their non-smoking counterparts. In a study by Jacobsen et al. (2007) adolescent smokers were allowed to smoke during a midway break on an attention task, and they still performed worse than non-smokers.

The dichotomy in the beneficial effects of nicotine and related compounds is probably due to alterations in the cholinergic and/or cortical attention system of smokers and patients with distinct psychiatric diseases. Various factors maybe causing this. For instance, it might relate to some extent to smoking-induced changes in nicotinic receptor number and sensitivity (Kadir et al., 2006; Brasic et al., 2012), or alternatively, disease-specific developmental disturbances in receptor expression or aberrant development of circuitry may underlie this.

In smokers, whose nicotinic acetylcholine receptor signaling pathways have undergone adaptations due to chronic nicotine exposure, nicotine can be beneficial for attention performance. However, this beneficial effect of nicotine is in the context of generally impaired attention and cognitive ability after nicotine deprivation (Kleykamp et al., 2011; Vossel et al., 2011). In fact, the effect of nicotine, as that of many other drugs, resembles an inverted U-shape function in which subjects who perform at suboptimum levels will show increased performance after drug stimulation (Newhouse et al., 2011). Thus, smoking or nicotine administration in nicotine-dependent smokers only reverses the impairment in cognitive function caused by abstinence from smoking (Sacco et al., 2004).

This difference in altered state of nicotine sensitive pathways is reflected in studies in which compounds, directly or indirectly targeting nAChRs, are tested in (pre)clinical trials for their cognitive enhancing effects. One of these, AZD3480/TC1734, a partial agonist of $\alpha4\beta2^*$ nAChR, exhibits memory-enhancing properties in healthy rodents and man (Obinu et al., 2002; Dunbar et al., 2007), albeit with mixed results in different patient populations (Dunbar et al., 2011; Frolich et al., 2011; Velligan et al., 2012). Despite initial positive reports on the effects in a phase II clinical trial, in which AZD3480 seemingly improved symptoms of ADHD, as well as results on the Stop Signal Reaction task, no further publication has yet appeared. In rats however, AZD3480 has been shown to improve MK801-induced impairments in accuracy as measured in an operant signal detection task, without having an effect on its own (Rezvani et al., 2012). Thus, novel drugs targeting the nicotinic receptors might have beneficial effects in a brain in which cholinergic signaling is disturbed (**Figure 1**).

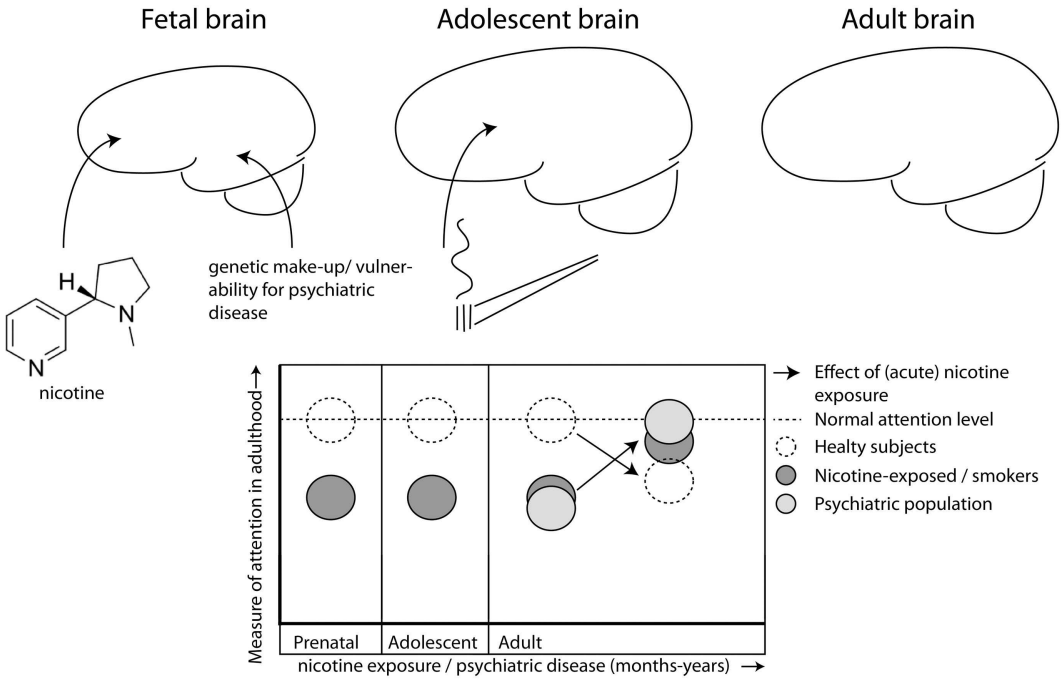

FIGURE 1 | Nicotine exposure during prenatal and/or adolescent development has long-lasting detrimental effects on attention, even after prolonged abstinence. Exposure during both developmental periods (dark gray) has additive effects leading to worse performance than healthy controls (open, hatched line) during adulthood. Patients with certain psychiatric disorders (e.g., ADHD, schizophrenia; light gray) also suffer from decreased attention performance. Nicotine has beneficial effects on attention, but only in those individuals who have decreased levels of attention to begin with, like smokers (Lawrence et al., 2002) and patient populations (Lerman et al., 2001; Newhouse et al., 2004), but not in healthy controls (hatched line; Levin et al., 1997, 1998; Wignall and de Wit, 2011), albeit that nicotine is not always beneficial for smokers (Jacobsen et al., 2005, 2007).

FUTURE PERSPECTIVES

Nicotine acts in the brain via a complex repertoire of receptor subtypes. Not surprisingly, many of the precise underlying mechanisms of action of nicotine on cognitive function still need to be revealed. Experimental work on animal models might assist in this.

Results from clinical and preclinical studies show that efficacy of nicotinic receptor targeting drugs could in principle be profoundly influenced by differences in the state of the cholinergic system and/or that of the involved circuitry, and hence possibly the health or disease status of the individual. Nicotinic-compounds are promising as cognitive enhancers, and most likely act only in patients. This has the consequence that the type of animal model used to screen treatment efficacy should well fit the disease state targeted. Modeling such a state at the preclinical level can be achieved with genetic alterations, or by using pharmacological agents. Nicotine exposure during specific developmental periods maybe one of these, thereby assuring that stable alterations in receptor levels or signaling state of patients is mimicked. In addition, studying animals carrying human gene mutations offers the possibility of specifically addressing the functional role in behavioral output related to the human disease (Trueman et al., 2012). Moreover, it should be noted that "cognitive improvement" is a broad concept, and that preclinical models should try to address a large panel of behavioral phenotypes, including altered states of attentional performance, which are in general more difficult to address. Current improvements in technology and animal behavioral paradigms hold a promise for the further mechanistic understanding of the effects of nicotine on the brain (Endo et al., 2011; Winter and Schaefers, 2011).

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Re-evaluation of nicotinic acetylcholine receptors in rat brain by a tissue-segment binding assay

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Nicotinic acetylcholine receptors (nAChRs) of the cerebral cortex and cerebellum of rats were evaluated by a radioligand binding assay, employing tissue segments, or homogenates as materials. [^{3}H]-epibatidine specifically bound to nAChRs in rat cortex or cerebellum, but the dissociation constants for [^{3}H]-epibatidine differed between segments and homogenates (187 pM for segments and 42 pM for homogenates in the cortex and 160 pM for segments and 84 pM for homogenates in the cerebellum). The abundance of total nAChRs was approximately 310 fmol/mg protein in the segments of cortex and 170 fmol/mg protein in the segments of cerebellum, which were significantly higher than those estimated in the homogenates (115 fmol/mg protein in the homogenates of the cortex and 76 fmol/mg protein in the homogenates of the cerebellum). Most of the [^{3}H]-epibatidine binding sites in the cortex segments (approximately 70% of the population) showed high affinity for nicotine ($pK_i = 7.9$), dihydro-β-erythroidine, and cytisine, but the binding sites in the cerebellum segments had slightly lower affinity for nicotine ($pK_i = 7.1$). An upregulation of nAChRs by chronic administration of nicotine was observed in the cortex segments but not in the cerebellum segments with [^{3}H]-epibatidine as a ligand. The upregulation in the cortex was caused by a specific increase in the high-affinity sites for nicotine (probably α4β2). The present study shows that the native environment of nAChRs is important for a precise quantitative as well as qualitative estimation of nAChRs in rat brain.

Keywords: nicotinic receptor, tissue-segment binding, upregulation

INTRODUCTION

Nicotinic acetylcholine receptors (nAChRs) in the nervous system are members of a family of ligand-gated ion channels, pentametric in structure, with at least 11 different subunits (α2–α10 and β2–β4) characterized (Lindstrom et al., 1996; Alexander et al., 2009). Because different subunit compositions produce distinct nAChR subtypes, the potential nAChR subtype diversity is vast (Lindstrom et al., 1996). However, in general, α4β2 and α7 are the predominant subtypes expressed in the central nervous system (CNS), whereas the α3β4 subtype is the predominant subtype expressed in the autonomic ganglia (Lindstrom, 2000; Alexander et al., 2009).

The nAChRs in the CNS are physiologically relevant in a wide range of neuronal functions (such as cognition, motor control, analgesia, and reward), and may be involved in pathological states such as Alzheimer's and Parkinson's diseases (Grady et al., 2007; Quik et al., 2007; Drever et al., 2010). Chronic nicotine exposure, including smoking, is known to increase the number of nAChRs in the CNS of rodents and humans, although there is a region-specific variation in the upregulation (Marks et al., 1992; Sanderson et al., 1993; Davil-Garcia et al., 2003; Govind et al., 2009). Thus, smoking seems to have certain therapeutic effects for Alzheimer's disease, although this conclusion is controversial (Hellstrom-Lindahl et al., 2004; Connelly and Prentice, 2005; Picciotto and Zoli, 2008). In addition, nAChRs have recently attracted attention as a novel target of general anesthetics and antidepressants (McMorn et al., 1993; Shytle et al., 2002; Tassonyi et al., 2002; Picciotto et al., 2008).

Identifying individual nAChR subtypes in the CNS has been a difficult task. The "high-affinity agonist binding" α4β2 subtype (labeled by [^{3}H]-acetylcholine, [^{3}H]-cytisine, (−)-[^{3}H]-nicotine) and the predominantly or entirely α7 subtype (labeled by [^{125}I]-?α-bungarotoxin) have been extensively characterized (Whiting and Lindstrom, 1987; Schoepfer et al., 1990; Flores et al., 1992; Picciotto et al., 1995; Orr-Urtreger et al., 1997; Marubio et al., 1999). Subsequently, the discovery of epibatidine (an azabicycloheptane alkaloid isolated from the skin of the Ecuadorian frog *Epipedobates tricolor*) that binds to multiple nAChR subtypes with high-affinity provided a tool to identify novel nAChR subtypes (Houghtling et al., 1995; Perry and Kellar, 1995; Marks et al., 1998; Whiteaker et al., 2000; Grady et al., 2007). In the CNS of rodents, the majority of high-affinity [^{3}H]-epibatidine binding sites represent the α4β2 subtype, but the minority of the sites were distinguished by their relatively low-affinity for cytisine or

dihydro-β-erythroidine in small nuclei dispersed across the brain (Grady et al., 2007).

In most studies of nAChRs and other receptors in the CNS, brain tissue was homogenized, and the resulting membrane or particulate preparations have been exclusively used in the radioligand binding assay (Bylund and Toews, 1993; Houghtling et al., 1995; Yang et al., 1998; Marubio et al., 1999; Whiteaker et al., 2000; Schneider and Michel, 2010). Although such a grind-and-bind approach has been successfully used to identify receptor pharmacology for more than two decades, tissue homogenization may cause alterations in receptor environments, resulting in different properties from the inherent profile in some receptors (Hiraizumi-Hiraoka et al., 2004; Anisuzzaman et al., 2008, 2011; Morishima et al., 2008). These recent studies suggest that the *in situ* environment significantly contributes to some receptor properties. To avoid artificial modification of the receptor environment as much as possible, a binding assay for use with intact tissue segments and with physiological solution (such as Krebs solution) was recently developed and has been demonstrated to be a powerful method for detecting the inherent profiles of receptors in a native manner (Tanaka et al., 2004; Muramatsu et al., 2005; Yoshiki et al., 2009; Anisuzzaman et al., 2011). In contrast to the conventional membrane binding assay, the tissue-segment binding assay is less prone to have a yield loss of receptors and to have a modification of the natural environment/conformation of receptors, which may be caused during tissue homogenization and membrane fractionation.

Previously, we detected a difference in [^{3}H]-epibatidine binding in the rat cerebral cortex between the tissue-segment binding assay and the conventional membrane binding assay (Muramatsu et al., 2005). In this study, we extensively evaluated [^{3}H]-epibatidine binding to nAChRs in the intact segments of rat cerebral cortex and cerebellum, and compared the pharmacological profiles with those in the homogenized preparations. Furthermore, the effects on nAChRs of chronic nicotine administration were examined by the tissue-segment binding assay.

MATERIALS AND METHODS

ANIMALS

Male Wistar rats weighing 250–350 g (SLC, Shizuoka, Japan) were used. The present study was performed according to the Guidelines for Animal Experiments, University of Fukui (which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology, Japan).

TISSUE-SEGMENT BINDING ASSAY

Rats were anesthetized with pentobarbital and killed by cervical dislocation. The brain was rapidly isolated and immersed in modified Krebs–Henseleit solution containing: NaCl, 120.7 mM; KCl, 5.9 mM; $MgCl_2$, 1.2 mM; $CaCl_2$, 2.0 mM; NaH_2PO_4, 1.2 mM; $NaHCO_3$, 25.5 mM; and D-(+)-glucose, 11.5 mM (pH = 7.4). This solution had been oxygenated with a mixture of 95% O_2 and 5% CO_2 and was kept at 0°C. The brain was cleaned from the pia mater and substantia alba, and then the cerebral cortex and cerebellum were cut into small pieces (approximately 2 mm × 2 mm × 1 mm). The tissue-segment binding assay was performed at 4°C for 26–30 h, according to the method previously described (Muramatsu et al., 2005; Anisuzzaman et al., 2011). Each segment of the cortex or cerebellum was incubated in 0.5 ml of the modified Krebs–Henseleit solution.

In saturation binding experiments, [^{3}H]-epibatidine was used at concentrations ranging from 30 to 2000 pM. In competition experiments, 400 pM [^{3}H]-epibatidine competed against various concentrations of tested drugs. After incubation at 4°C, tissue segments were quickly moved into a plastic tube containing 1.5 ml of ice-cold (4°C) modified Krebs–Henseleit solution and carefully washed by vortexing for 1 min. By this procedure, most of the unbound radioligand was released from the segments into the buffer medium and adsorbed to the plastic tube. The segments were then solubilized in 0.3 M NaOH solution to estimate the bound radioactivity and protein content. Non-specific binding was determined in the presence of 100 μM nicotine. The bound radioactivity was measured using a liquid scintillation counter (Aloka, Tokyo, Japan). Protein concentration in each tissue segment was measured by the Bio-Rad commercial protein assay. Experiments were done in duplicate at each concentration of [^{3}H]-epibatidine for binding saturation experiments or at each concentration of competing ligand for competition binding experiments.

HOMOGENATE BINDING ASSAY

As reported previously (Anisuzzaman et al., 2011), the rat cerebral cortex and cerebellum were homogenized in 40 volumes (v/w; for cortex) or 20 volumes (v/w; for cerebellum) of the modified Krebs–Henseleit solution using a Polytron homogenizer at 4°C. The resulting homogenates were incubated with [^{3}H]-epibatidine in a final volume of 0.5 ml modified Krebs–Henseleit solution for 5 h at 4°C. In competition experiments, 200 pM (for cortex) and 300 pM (for cerebellum) of [^{3}H]-epibatidine were used. Non-specific binding of [^{3}H]-epibatidine was determined with 100 μM nicotine. The assay was terminated by rapid filtration over Whatman GF/C filters presoaked with 0.3% polyethyleneimine using a Brandel cell harvester and filters were rapidly washed with 5-ml aliquots of ice-cold modified Krebs–Henseleit solution. The filters were then dried and the radioactivity retained on the filter paper was measured by liquid scintillation counting. The protein contents of the homogenates were determined by the Bio-Rad commercial protein assay.

CHRONIC ADMINISTRATION OF NICOTINE

Nicotine was administered for 3 weeks in drinking water. The concentration of nicotine in the drinking water was 50 μg/ml. The control rats drank tap water during the experimental period. The plasma concentration of nicotine after 3 weeks of oral nicotine exposure was measured with high performance liquid chromatography; the plasma level was 18 ± 3 ng/ml ($n = 5$), which was similar to the nicotine concentrations (10–50 ng/ml) reported in smokers (Benowitz et al., 1982; Pekonen et al., 1993).

After nicotine administration for 3 weeks, rats were killed and the cortex or cerebellum segments were used in the binding experiments, as described above.

DATA ANALYSIS

Binding data were analyzed using PRISM software (Version 5.01, Graph Pad Software, La Jolla, CA, USA), as previously described

(Muramatsu et al., 2005; Yoshiki et al., 2009). Briefly, the data from saturation binding studies were fitted by a one-site saturation binding isotherm and the K_d values and the binding capacity were then calculated. The abundance of the nAChRs is indicated as the maximum binding capacity per milligram of total tissue protein (Bmax). For the competition studies, the data were analyzed using the Binding-Competitive Equation of the PRISM software. A two-site model was adopted only when the residual sums of squares were significantly less ($P < 0.05$) for a two-site fit to the data than for a one-site by F test comparison.

Data are represented as the mean ± SEM with the number of experiments (n). Results were considered to be significant where P values were less than 0.05 (Student's t-test).

DRUGS

The following drugs were used in the present study: [5,6-bicycloheptyl-^{3}H]-epibatidine (specific activity, 2065 GBq/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA, USA); 1-quinuclidinyl-[phenyl-4-^{3}H]-benzilate ([^{3}H]-QNB; specific activity, 1.81 TBq/mmol; GE Healthcare, Buckinghamshire, UK); nicotine, nicotine tartrate dihydrate, and hexamethonium chloride (Nacalai Tesque, Kyoto, Japan); dihydro-β-erythroidine, cytisine, and mecamylamine hydrochloride (Tocris Cookson Ltd., Bristol, UK); and α-bungarotoxin (Peptide Institute, Osaka, Japan). For chronic administration of nicotine, aqueous nicotine was used.

RESULTS

[^{3}H]-EPIBATIDINE BINDING IN RAT CEREBRAL CORTEX AND CEREBELLUM

The representative time courses of [^{3}H]-epibatidine binding to the intact segments (**Figure 1A**) and homogenate preparations (**Figure 1B**) of rat cerebral cortex and to the intact segments (**Figure 2A**) and homogenate preparations (**Figure 2B**) of rat cerebellum are shown. At 4°C, the binding to the segments was extremely slow compared with that in the homogenates; therefore, different incubation periods were applied in the segment binding assay (26–30 h) and the homogenate binding assay (5 h). In the saturation experiments, the specific binding of [^{3}H]-epibatidine to both the intact segments (**Figure 1C**) and homogenate preparations (**Figure 1D**) of rat cortex was saturable at the concentrations tested and fitted a single-site model. However, the slopes of the saturation curves were apparently different; the slope was steeper for the homogenates than for the segments. Thus, the dissociation constant (K_d) for [^{3}H]-epibatidine was estimated to be four times higher in the segment binding assay than in the homogenate binding assay (**Table 1**). Furthermore, the maximum binding capacity (Bmax) of [^{3}H]-epibatidine was also approximately three times higher in the segments than in the homogenates of rat cortex (**Table 1**). Differences in the time course, the density, and the dissociation constant for [^{3}H]-epibatidine binding were also observed between the segments and homogenates of rat cerebellum (**Figure 2** and **Table 1**). The affinity for [^{3}H]-QNB (a

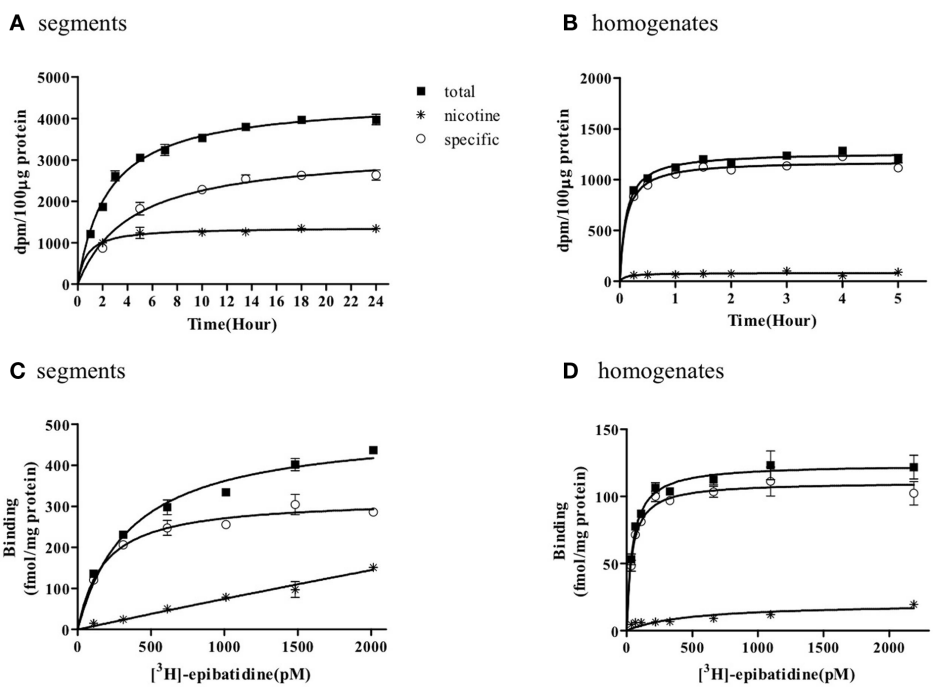

FIGURE 1 | Time course and saturation isotherm of [^{3}H]-epibatidine binding in the intact segments (A,C) and homogenates (B,D) of rat cerebral cortex. Time course **(A,B)**: The intact segments were incubated with 1000 pM [^{3}H]-epibatidine at 4°C for 1–24 h and the homogenates were incubated with 500 pM [^{3}H]-epibatidine at 4°C for 15 min to 5 h. Saturation binding **(C,D)**: The intact segments and homogenates were incubated with [^{3}H]-epibatidine (up to 2000 pM) at 4°C for 28 h (segments) or 5 h (homogenates). Specific binding (open circles) was determined by subtracting the amount bound in the presence of 100 μM nicotine (asterisks) from total binding (closed squares). Each point represents the mean of duplicate determinations. Each figure represents four to five experiments.

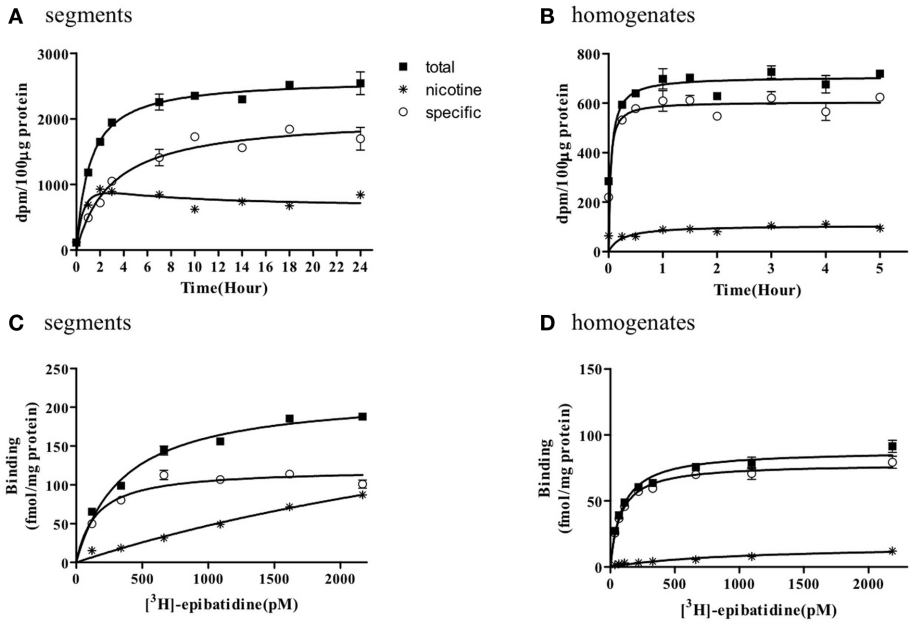

FIGURE 2 | Time course and saturation isotherm of [^{3}H]-epibatidine binding in the intact segments (A,C) and homogenates (B,D) of rat cerebellum. Time course **(A,B)**: The intact segments were incubated with 1000 pM [^{3}H]-epibatidine at 4˚C for 1–24 h and the homogenates were incubated with 500 pM [^{3}H]-epibatidine at 4˚C for 15 min to 5 h. Saturation binding **(C,D)**: The intact segments and homogenates were incubated with [^{3}H]-epibatidine (up to 2000 pM) at 4˚C for 28 h (segments) or 5 h (homogenates). Specific binding (open circles) was determined by subtracting the amount bound in the presence of 100 μM nicotine (asterisks) from total binding (closed squares). Each point represents the mean of duplicate determinations. Each figure represents four to five experiments.

Table 1 | Binding parameters of [^{3}H]-epibatidine and [^{3}H]-QNB in rat cortex and cerebellum.

Tissue	Radioligand	Segments		Homogenates	
		K_d(pM)	Bmax	K_d(pM)	Bmax
Cortex	[^{3}H]-epibatidine	187 ± 20^a	313 ± 6^a	42 ± 10^a	115 ± 13^a
	(Nicotine-treated)	$(388 \pm 15)^{a,b}$	$(632 \pm 25)^{a,b}$	$(163 \pm 8)^{a,b}$	$(179 \pm 14)^{a,b}$
	[^{3}H]-QNB	1237 ± 104^a	2354 ± 189	285 ± 56^a	2287 ± 105
Cerebellum	[^{3}H]-epibatidine	160 ± 34^a	172 ± 12^a	84 ± 3^a	76 ± 8^a

Segment and homogenates binding experiments with [^{3}H]-epibatidine and [^{3}H]-QNB were conducted at 4˚C in the cortex and cerebellum of nicotine-untreated rats. [^{3}H]-epibatidine binding in the cortex of nicotine-treated rats was also represented in parenthesis.

Data represents mean ± SEM of four to six experiments. Bmax: fmol/mg tissue protein.

[a] *Significantly different between segments and homogenates ($P < 0.05$).*

[b] *Significantly different from control and nicotine-treated rats ($P < 0.05$).*

muscarinic receptor ligand that was used as control) was also lower in the cortex segments than in the homogenates, but the binding capacity was not significantly different between the segments and the homogenates (**Table 1**).

Figures 1 and **2** further demonstrate that non-specific binding of [^{3}H]-epibatidine was significantly higher in the segments than in the homogenates. The non-specific binding in the segments was rapidly equilibrated compared with specific binding (**Figures 1A** and **2A**) and increased lineally with increasing concentrations of [^{3}H]-epibatidine (**Figures 1C** and **2C**). Because the non-specific binding was insensitive to not only nicotine but also the other tested ligands (see the competition experiments), it was considered that there was a significant amount of non-nAChR sites in the segments that were undetectable after homogenization.

Because the binding capacity and dissociation constant for [^{3}H]-epibatidine were significantly different between tissue segments and homogenates, the pharmacological profiles of [^{3}H]-epibatidine binding sites in both preparations of rat cortex were compared in competition binding experiments using various drugs. Nicotine, dihydro-β-erythroidine, and cytisine showed shallow competition curves in the segments of cortex and approximately 70% of the specific binding sites were identified

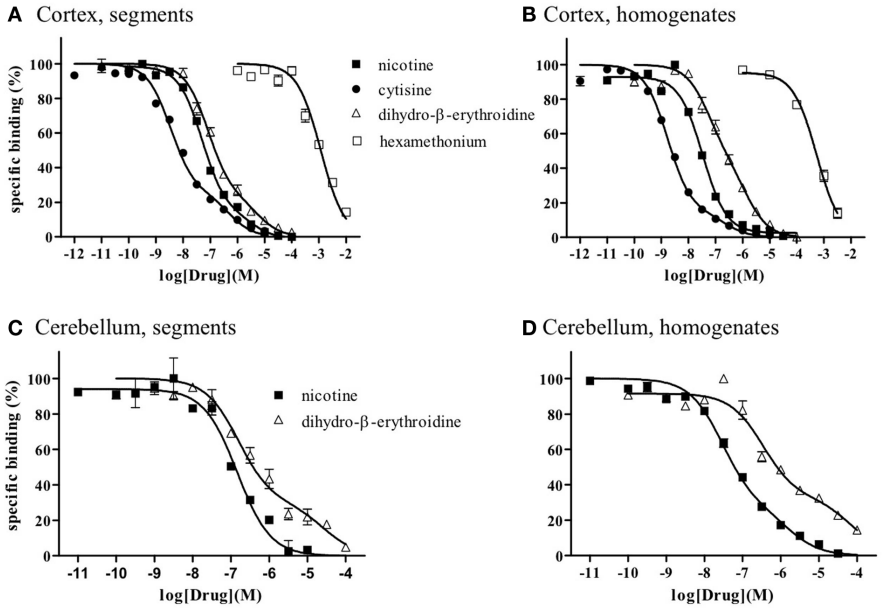

FIGURE 3 | Competition curves for various dugs at [^{3}H]-epibatidine binding sites in intact segments and homogenates of rat cerebral cortex (A,B) and rat cerebellum (C,D). Intact segments **(A,C)** were incubated with 400 pM [^{3}H]-epibatidine for 28 h at 4˚C. Homogenates were incubated with 200 pM [**(B)**: cortex] or 300 pM [**(D)**: cerebellum] [^{3}H]-epibatidine for 5 h at 4˚C. Each point represents the mean of duplicate determinations. Each figure represents four to five experiments.

as high-affinity sites for these ligands (**Figure 3A**). In the homogenates, dihydro-β-erythroidine and cytisine also showed shallow curves, but the competition curve for nicotine better fitted a one-site model with a slightly higher pK_i value than that in the segments (**Figure 3B** and **Table 2**). Hexamethonium at concentrations higher than 10 μM were competitive, but mecamylamine (1 mM) and α-bungarotoxin (1–30 nM) failed to compete in the [^{3}H]-epibatidine binding assay with both segment and homogenate preparations (**Table 2**). In general, the estimated ligand affinities were higher in the homogenates compared with the segments.

Because of the tissue limitation, the pharmacological profile at [^{3}H]-epibatidine binding sites in the cerebellum was examined with nicotine and dihydro-β-erythroidine. **Figure 3** shows the competition curves in the segments (**Figure 3C**) and the homogenates (**Figure 3D**) of rat cerebellum. Nicotine showed biphasic competition in the homogenates (pKi = 8.2 and 6.3), but competed for binding to the segments monotonically with an intermediate affinity (pKi = 7.1). These results are summarized in **Table 2**.

Table 2 | Pharmacological characteristics of [^{3}H]-epibatidine binding sites in rat cortex and cerebellum.

Drugs (selectivity)	Segments		Homogenates	
	p$K_{i_{high}}$ (%)	p$K_{i_{low}}$ (%)	p$K_{i_{high}}$ (%)	p$K_{i_{low}}$ %)
CORTEX				
Nicotine	7.9 ± 0.1	6.5 ± 0.1	8.4 ± 0.1	
(α4β2 > α4β4)	(72%)	(28%)	(100%)	
Dihydro-β-erythroidine	7.1 ± 0.1	5.7 ± 0.2	7.7 ± 0.2	6.3 ± 0.1
(α4β2, α4β4)	(69%)	(31%)	(66%)	(34%)
Mecamylamine	NI		NI	
Cytisine	8.7 ± 0.1	6.7 ± 0.1	9.4 ± 0.0	7.2 ± 0.2
(α4β2, α4β4)	(73%)	(27%)	(85%)	(15%)
Hexamethonium	3.4 ± 0.1		4.0 ± 0.1	
	(100%)		(100%)	
α-Bungarotoxin	NI		NI	
(α7, α9)				
CEREBELLUM				
Nicotine	7.1 ± 0.2		8.2 ± 0.1	6.3 ± 0.1
(α4β2 > α4β4)	(100%)		(68%)	(32%)
Dihydro-β-erythroidine	7.4 ± 0.3	5.1 ± 0.3	7.1 ± 0.1	4.6 ± 0.2
(α4β2, α4β4)	(70%)	(30%)	(67%)	(33%)

%, Proportion of high or low-affinity sites. Mean ± SE of four to five rats. NI, no inhibition.

Selectivity: subtype selectivity at concentrations around 10 nM.

EFFECTS OF CHRONIC ADMINISTRATION OF NICOTINE ON [^{3}H]-EPIBATIDINE BINDING SITES

Chronic administration of nicotine is known to cause an increase in nAChRs in the brain, which is called upregulation. Because the number of [^{3}H]-epibatidine binding sites was underestimated in homogenates as mentioned above, the nicotine-induced upregulation was re-evaluated by the tissue-segment binding approach. Administration of nicotine for 3 weeks increased the binding density of [^{3}H]-epibatidine with a slight decrease in affinity in the rat cortex segments (**Table 1**). A similar tendency was observed in the cortex homogenates, but the density and affinity were also significantly different from the values estimated in the

cortex segments of chronically nicotine-treated rats (**Table 1**). The binding affinities for nicotine in nicotine-treated rat cortex (pKi_{high} and $pKi_{low} = 7.8 \pm 0.2$ and 6.3 ± 0.2 in the segments and $pKi = 8.1 \pm 0.1$ in the homogenates) were not significantly different from those in rats not treated with nicotine (**Table 2**). However, the proportion of nicotine-high-affinity sites in the cortex segments increased from 72% in control rats to 86% in nicotine-treated rats (**Figure 4**). On the other hand, no upregulation was caused by chronic nicotine administration in the cerebellum.

DISCUSSION

In the binding study with [^{3}H]-epibatidine, the nAChRs in the rat cerebral cortex and cerebellum were evaluated and compared between the segments and the homogenates. Two major differences were observed in the present study. The first is different affinities for [^{3}H]-epibatidine and other nicotinic ligands between the two preparations. Previous reports with particulate preparations of rat cortex showed an extremely high affinity for [^{3}H]-epibatidine (K_d = approximately 20 pM; Houghtling et al., 1995; Marks et al., 1998). In the present study as well, such a high affinity for [^{3}H]-epibatidine was estimated in the cortex homogenates ($K_d = 42$ pM), whereas the affinity in the segments was significantly low ($K_d = 187$ pM). Consistent with this difference in affinity, the binding time course of [^{3}H]-epibatidine was faster in the homogenates than in the segments.

It has been classically considered that antagonist affinities are constant for a given receptor subtype, regardless of the cell/tissue background in which the receptor is expressed; this has been called "antagonist assumption." However, this traditional view has now been challenged by the observation of different pharmacological antagonist profiles for the same gene product in different cells/tissues (Kenakin, 2003; Baker and Hill, 2007; Nelson and Challiss, 2007; Muramatsu et al., 2008; Nishimune et al., 2011). The present results also suggest that the pharmacological properties of nAChRs are not necessarily constant, but rather may be easily modified by assay conditions and probably by distinct receptor environments.

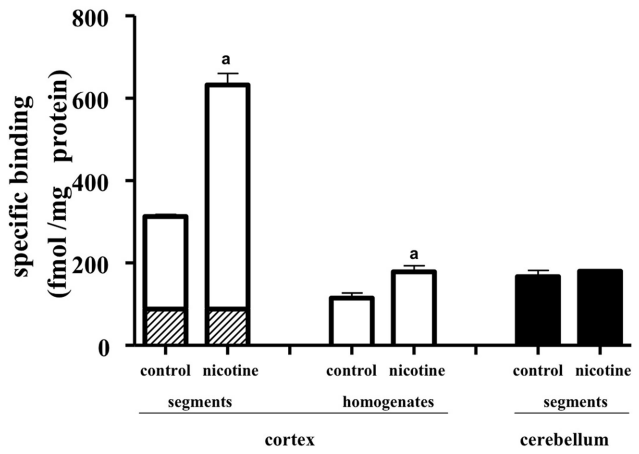

FIGURE 4 | Effects of chronic administration of nicotine for 3 weeks. The tissue segments or homogenates of the cerebral cortex and cerebellum in nicotine-treated and untreated rats were incubated with [^{3}H]-epibatidine for 28 h (segments) or 5 h (homogenates) at 4°C. The total density of [^{3}H]-epibatidine binding sites was estimated from saturation experiments in the cortex and from 2 nM [^{3}H]-epibatidine binding in the cerebellum. From the competition curves for nicotine, high-affinity (open column), and low-affinity sites (hatched column) for nicotine were calculated in the cerebral cortex. The binding sites in the cerebellum were not characterized by nicotine and were represented as black columns. Control, rats not treated with nicotine; nicotine, nicotine-treated rats. Mean ± SEM of four experiments. a: Significantly different from those of the control rats.

In general, there is a tendency for antagonist affinity to become higher after homogenization, even though the other experimental conditions were the same. The tendency was observed in the binding of [^{3}H]-epibatidine to nAChRs (present study) and [^{3}H]-QNB or [^{3}H]-*N*-methylscopolamine to muscarinic AChRs (present results and the results of Anisuzzaman et al., 2011). Although the precise mechanisms underlying the change in pharmacological profile by homogenization remain to be solved, intact tissue can demonstrate distinct submembrane effector interactions in the presence of constrained membrane architecture, which may be easily destroyed by homogenization. In general, the relatively universal high-affinity obtained in the classical grind-and-bind approach seems to be one of the bases of "antagonist assumption."

The second difference observed in the present study is distinct densities of nAChRs estimated between segments and homogenates. This is unique for nAChRs ([^{3}H]-epibatidine binding sites), because the abundance of muscarinic AChRs ([^{3}H]-QNB binding sites) did not change after homogenization (**Table 1**). However, it has been generally reported that homogenization causes a yield loss of receptors (Colucci et al., 1981; Kwan et al., 1981; Faber et al., 2001; Tanaka et al., 2004; Su et al., 2008). The density of [^{3}H]-epibatidine binding sites estimated in the present segment study with rat cortex (313 fmol/mg tissue protein) is greater than the estimates reported previously in conventional membrane binding assays with rat cortex (5–83 fmol/mg of membrane protein; Houghtling et al., 1995; Flores et al., 1997; Davil-Garcia et al., 2003) and with mouse cortex (54.9 fmol/mg of membrane protein; Marks et al., 1998). Previously, we showed that the protein yield of crude membrane preparations in rat cortex after simple centrifugation or fractionation was approximately 50% of the total tissue protein (Morishima et al., 2008). Therefore, it seems that the density of nAChRs estimated previously in the concentrated membrane preparations may be much lower, if the values were adjusted at the same denominator level (that is, per mg of "total tissue protein").

As mentioned above, the present study reveals that intact tissue conditions or the native physiological environment are very important in the quantitative and qualitative evaluation of nAChRs. However, we are not neglecting the classical grind-and-bind approach and recognize its successful and significant contribution to receptor pharmacology over the past two decades. Indeed, the present competition study with segments and homogenates clearly shows high- and low-affinity sites for dihydro-β-erythroidine and cytisine in the [^{3}H]-epibatidine binding sites, which is consistent with the previous results obtained in membrane binding studies (Whiteaker et al., 2000; Grady et al., 2007). The high-affinity sites in the cortex are major in proportion and seem to correspond to α4β2 and/or α4β4, as reported previously.

In the segments, the [^{3}H]-epibatidine binding sites were composed of high- and low-affinity sites for nicotine in the cortex but a single affinity site in the cerebellum. It is interesting to note that the high affinity for nicotine in the cortex (pKi = 7.9) was slightly higher than the affinity in the cerebellum (pKi = 7.1). Because plasma concentrations of nicotine are reported to be 10–50 ng/ml in nicotine-treated rats and in smokers (present study; Benowitz et al., 1982; Pekonen et al., 1993), the high-affinity sites for nicotine in the cortex may mainly contribute to the upregulation, as shown in **Figure 4**. The extent of upregulation estimated in the cortex segments (nearly 100%) was greater than those reported in previous membrane binding studies and in the present data of homogenate binding (approximately 50%). On the other hand, no upregulation was observed in the cerebellum in the present segment binding assay as well as previous membrane binding assays (Marks et al., 1992; Flores et al., 1997; Pietila et al., 1998), confirming regional variation of the nicotine-induced upregulation.

In summary, the present comparative binding study with tissue segments and homogenates shows that the native conformation and inherent properties of nAChRs are strongly modified by the receptor environment and suggests that keeping intact tissue conditions in the assays is important for determining the properties of nAChRs and probably other receptors.

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Nicotine, auditory sensory memory, and *sustained* attention in a human ketamine model of schizophrenia: moderating influence of a hallucinatory trait

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Background: The procognitive actions of the nicotinic acetylcholine receptor (nAChR) agonist nicotine are believed, in part, to motivate the excessive cigarette smoking in schizophrenia, a disorder associated with deficits in multiple cognitive domains, including low-level auditory sensory processes and higher-order attention-dependent operations. **Objectives:** As *N*-methyl-d-aspartate receptor (NMDAR) hypofunction has been shown to contribute to these cognitive impairments, the primary aims of this healthy volunteer study were to: (a) to shed light on the separate and interactive roles of nAChR and NMDAR systems in the modulation of auditory sensory memory (and *sustained* attention), as indexed by the auditory event-related brain potential – mismatch negativity (MMN), and (b) to examine how these effects are moderated by a predisposition to auditory hallucinations/delusions (HD). **Methods:** In a randomized, double-blind, placebo-controlled design involving a low intravenous dose of ketamine (0.04 mg/kg) and a 4 mg dose of nicotine gum, MMN, and performance on a rapid visual information processing (RVIP) task of sustained attention were examined in 24 healthy controls psychometrically stratified as being lower (L-HD, $n = 12$) or higher (H-HD) for HD propensity. **Results:** Ketamine significantly slowed MMN, and reduced MMN in H-HD, with amplitude attenuation being blocked by the co-administration of nicotine. Nicotine significantly enhanced response speed [reaction time (RT)] and accuracy (increased % hits and d' and reduced false alarms) on the RVIP, with *improved performance accuracy* being prevented when nicotine was administered with ketamine. Both % hits and d', as well as RT were poorer in H-HD (vs. L-HD) and while hit rate and d' was increased by nicotine in H-HD, RT was slowed by ketamine in L-HD. **Conclusions:** Nicotine alleviated ketamine-induced sensory memory impairment and improved attention, particularly in individuals prone to HD.

Keywords: nicotine, nicotinic acetylcholine receptor, *N*-methyl-d-aspartate receptor, sensory memory, mismatch negativity, attention, rapid visual information processing task, schizophrenia

INTRODUCTION

In patients with schizophrenia, the cardinal clinical (positive and negative) symptoms are, to variable degrees, accompanied by a wide range of neurocognitive impairments, particularly in the domains of attention and working memory. The various cognitive deficits are neither state-related nor specific to subtypes of the illness, but are considered as enduring features which remain relatively constant over the course of the disease and are at the very core of the dysfunction in schizophrenia patients (Elevåg and Goldberg, 2000; Heinrichs, 2005). As cognitive impairments in schizophrenia are highly correlated with functional (social and vocational) outcome, and both are relatively unaffected by dopamine-based antipsychotic drugs, pharmacotherapeutic initiatives are focusing on alternative molecular mechanisms as promising pharmacological targets for improving cognitive abilities in this disorder (Green, 2007; Tcheremissine et al., 2012).

Converging evidence from preclinical and human research points to the therapeutic potential of drugs targeted at nicotinic acetylcholine receptors (nAChRs) in the treatment of schizophrenia cognition (Ochoa and Lasalde-Dominicci, 2007; Evans and Drobes, 2009; D'Souza and Markou, 2011). nAChRs are strongly implicated in normal cognitive information processes (Mansvelder et al., 2006). Nicotine, the primary psychoactive chemical in tobacco smoke, is a prototypical nAChR agonist and the increased prevalence of smoking in schizophrenia (∼70–80%) compared to the general population (∼20%) has been interpreted as a form of self-medication (Kumari and Postman, 2005; Winterer,

2010), perhaps to compensate for reduced expression of nAChRs observed in post-mortem hippocampal and cortical brain regions of patients (Breese et al., 2000). *Although recent evidence of nicotine interacting with schizophrenia risk genes with regards to the expression of endophenotypes such as sensory gating may suggest that smoking might act as a causal factor for schizophrenia and related cognitive deficits* (Quednow et al., 2012), meta-analysis has shown significant enhancing effects of acute nicotine on multiple cognitive domains in healthy smokers and non-smokers (Heishman et al., 2010). Although null findings have been reported in the cognitive literature, acute doses of nicotine in animal models of schizophrenia and in non-smoking and smoking schizophrenia patients also transiently improve elementary pre-attentive sensory processing deficits as well as performance impairments in higher-order attention and working memory tasks (Olincy and Stevens, 2007; Radek et al., 2010).

Neurochemical models of schizophrenia have *implicated* glutamatergic mechanisms in general and *N*-methyl-D-aspartate receptors (NMDARs) in particular. NMDAR-type glutamate receptors are widely distributed throughout the brain and single sub-anesthetic doses of NMDAR antagonists, such as phencyclidine or ketamine, induce transient and reversible neurochemical, symptomatic and neurocognitive aspects of the disorder in healthy controls, with information processing deficits being observed not only in higher cortical regions, but also in subcortical systems and sensory cortices (Javitt, 2010; Adell et al., 2012). Over the past decade, bottom-up sensory processing deficits have become increasingly well documented in schizophrenia, co-existing with top-down, more complex forms of cognitive impairments and mirroring the pattern of deficits seen with NMDAR antagonists, thus supporting a NMDAR hypofunction model of the disease (Kantrowitz and Javitt, 2010).

Disturbance in low-level sensory problems in the auditory system is a robust cognitive deficit in schizophrenia patients and is strongly indexed by impaired generation of an event-related brain potential (ERP) component – the mismatch negativity (MMN). Generated within the primary auditory cortex and receiving contributions from frontal cortical generators (Rinne et al., 2000), the MMN is automatically elicited (at ∼150–200 ms) in an auditory "oddball" paradigm in response to infrequent changes (i.e., deviants, such as shifts in sound pitch, intensity, duration, location, or pattern) in a repetitive stream of auditory stimuli. Presumed to reflect the stored representations of the characteristics of auditory stimuli (for seconds to minutes), MMN is an index of auditory sensory ("echoic") memory, a pre-attentive component of the brain working memory system (Naatanen et al., 2011). Impaired MMN generation, which is specific to schizophrenia (vs. other psychiatric disorders), is well established (Naatanen and Kahkonen, 2009), with a mean effect size of ∼1 d across studies (Umbricht and Krljes, 2005). Neither typical nor atypical antipsychotics affect the amplitude or latency of the ERP, and deficits in MMN generation have been reported in both clozapine- and risperidone-treated patients (Schall et al., 1998; Umbricht et al., 1998; Umbricht and Vollenweider, 1999; Kasai et al., 2002).

N-methyl-D-aspartate receptor antagonists have dose-dependently blocked the MMN response recorded in auditory primary cortex in animals (Javitt et al., 1994, 1996) and have diminished the MMN in healthy controls (Umbricht et al., 2000; Kreitschmann-Andermahr et al., 2001; Heekeren et al., 2008). Nicotine effects on MMN on the other hand have been relatively consistent with increased amplitude and shorter latencies to tone pitch deviants being observed in patients with Alzheimer' disease (Engeland et al., 2002), whereas in healthy controls, nicotine has augmented MMN amplitudes to pitch (Harkrider and Hedrick, 2005), inter-stimulus interval (Martin et al., 2009), and duration deviants (Baldeweg et al., 2006), with the two former MMN effects being observed both in smokers and non-smokers, while the latter effect was shown only in smokers. The latency of the pitch-MMN has also been shortened with nicotine administration in non-smokers (Inauri et al., 2005), who have also evidenced a shortened latency and an increased amplitude of the pitch-MMN with an acute dose of the selective nAChR agonist AZD3480 (Dunbar et al., 2007), as well as an amplitude increase of the visual MMN with nicotine (Fisher et al., 2010).

In the first of two studies in schizophrenia patients, acute nicotine treatment did not alter the pitch-MMN amplitude in non-smoking patients and controls, but in the latter group, it shortened MMN latency relative both to placebo and to the MMN latency seen in the patient group (Inami et al., 2007). In the second study, conducted in our laboratory, nicotine also increased duration- (but not pitch) MMN in smoking patients, normalizing their amplitude relative to control smokers (Dulude et al., 2010). These effects were not associated with tobacco withdrawal symptoms or antipsychotic medication.

Nicotine modulates the release of neurotransmitters other than acetylcholine (e.g., *dopamine*, GABA, norepinephrine, and *glutamate*), with presynaptic nAChRs facilitating NMDAR-mediated glutamatergic neurotransmission in a multitude of brain regions including (but not limited to) the prefrontal cortex (Lambe et al., 2003). *nAChRs are heavily expressed in the primary auditory cortex* (Soto et al., 2006) *where nicotine has enhanced tone-evoked physiological sensitivity through NMDAR activation* (Metherate, 2004). Previous preclinical cognitive studies of nAChR-NMDAR interactions have shown mixed findings (Timofeeva and Levin, 2011), with some reporting nicotine blockade of the disruptive pre-attentive (sensorimotor gating), attentional, and mnemonic effects of NMDAR antagonists (Thompson and Winsaver, 1986; Terry et al., 2002; Rezvani and Levin, 2003; Spieleway and Markov, 2004; Andreasen et al., 2006; Levin and Rezvani, 2006; Rezvani et al., 2008), while others reported no interactions, or observed a potentiation of impairments induced with NMDAR antagonist treatment (Levin et al., 2003, 2005; Rezvani and Levin, 2003; Quarta et al., 2007; Rasmussen et al., 2008). In the few human studies, the NMDAR antagonist memantine did not oppose the smoking-induced improvements in sustained attention (Jackson et al., 2009), and in our work with ketamine, nicotine moderation of the arousal and attentional modulating actions of this NMDAR antagonist were found to be dependent on smoker vs. non-smoker status (Knott et al., 2006, 2011).

Mismatch negativity deficits in schizophrenia are highly correlated with cognitive and functional outcome and as the NMDAR antagonist model has shown predictive ability for a range of novel treatments that have reached clinical trials (Large, 2007), the human ketamine model, together with the use of putative

endophenotypes such as MMN, offer a good opportunity to study new drugs with novel and distinct cognitive enhancing mechanisms that go beyond dopamine transmission. In the present study, the first to investigate the role of NMDAR-mediated glutamatergic neurotransmission in nicotine-modulated sensory memory, the separate and combined actions of nicotine and ketamine were examined with respect to auditory MMN. In addition, as NMDAR antagonism also impairs sustained attentional performance, typically slowing response speed and reducing accuracy in continuous performance tasks (CPT), a performance pattern similar to that observed in schizophrenia patients (Newcomer et al., 1999; Krystal et al., 2005a,b), the study also examined the effects of these drugs and their interactions on the Rapid Visual Information Processing (RVIP) task, a CPT with putative endophenotypic sensitivity (Hilti et al., 2010). As it is unclear as to whether ketamine-induced cognitive impairments reflect the direct effects of ketamine or are secondary to the induced schizophrenia-like clinical symptoms, we followed our previous studies and administered a sub-perceptual, non-psychotomimetic dose of ketamine (Knott et al., 2006, 2011).

Although the human ketamine model in healthy volunteers is well established and allows for the investigation of neurotransmitter systems participating in NMDAR-mediated cognitive deficiency, these findings may not necessarily be relevant to cognitive impairment in schizophrenia. Confounding factors (prior or concomitant drug treatment, chronicity, lack of cooperation, lower education) make the *in vivo* study of ketamine-drug interactions in schizophrenia difficult. However, the use of healthy surrogate populations (e.g., unaffected relatives of patients or people with schizotypal personality features), defined as groups that feature a component of the main disease process but do not have the fully developed condition – is gaining momentum as a potential methodology for detecting novel drug treatment for schizophrenia and as such may be relevant for capturing NMDAR-nicotinic interactions regulating cognitive endophenotypes of schizophrenia. In this approach, schizophrenia is viewed as an extreme of normally distributed cognitive functions and, for people who express some of the phenotype without the full disease (psychoses), their endophenotypes (such as MMN and RVIP) are thought to be more sensitive to drug effects and/or neurochemical disturbances than unselected healthy volunteers because they share the same elements of the disorder (Koychev et al., 2011). One phenotype, auditory hallucinations (AHs), is strongly associated with psychotic disorders such as schizophrenia but is also observed in other clinical and non-clinical groups (i.e., are "trans-diagnostic"). Increasingly, AHs are being investigated in non-psychotic conditions with cognitive and behavioral paradigms developed for schizophrenia (Waters et al., 2012). As the trait that makes humans prone to AHs appears to be related to brain areas involved in auditory stimuli processing/speech perception (i.e., auditory cortex; Kuhn and Gallinat, 2010), the same brain regions participating in MMN generation, and as schizophrenia patients with AHs (vs. those without AHs) have exhibited reduced MMN-indexed auditory sensory memory in our laboratory studies (Fisher et al., 2008, 2011, 2012a,b), this present investigation examined the effects of ketamine, nicotine, and their interactions in healthy volunteers psychometrically assessed as varying in hallucination/delusion (HD) proneness.

It was expected that individuals with higher (vs. lower) HD proneness would exhibit reduced MMN and RVIP performance, be more susceptible to the detrimental effects of ketamine, and be more responsive to the enhancing actions of nicotine when administered alone and in combination with ketamine.

MATERIALS AND METHODS

STUDY PARTICIPANTS

A sample of 24 participants (10 males, 14 females) was selected from a larger group of 38 healthy volunteers recruited from advertisements in local media and universities. All potential participants were screened via a semi-structured interview for psychiatric disorders (including alcohol/drug abuse) and general health, and were also assessed with the Family Instrument for Genetic Studies (Nurnberger et al., 1994) to rule out those with psychiatric disorders within first-degree relatives. They were also administered the Bell Object Relations and Reality Testing Inventory (BORRTI; Bell, 1992), a 90-item, self-report true-false pencil-and-paper questionnaire: 45 items assessing object relations, and the other 45 reality testing, with the latter yielding three subscale factors (Reality of Distortions, Uncertainty of Perceptions, and Hallucinations and Delusions), which are thought to identify those with a predisposition for psychotic symptoms. Only the HD subscale was used in this study. HD scores correlate significantly with the Hallucinatory Behavior and Unused Thought Content scales of the widely used Brief Psychiatric Rating Scale (BPRS; Overall and Gorham, 1962) and HD is the only subscale of the BORRTI on which high scores are specific to schizophrenia vs. other criterion groups (Bell et al., 1985). Standardized HD t scores of the total sample ranged from 30 to 67 (mean = 46.5) and those individuals with the 12 lowest (L-HD) scores (mean = 36.9, range 30–42) and the 12 highest (H-HD) scores (mean = 56.3, range 52–67) were selected for this study. A t score of 60 or more is indicative of a deficit and three of the H-HD group exhibited scores of 61, 61, and 67. L-HD and H-HD groups did not differ with respect to their scores on the Reality of Distortions and Uncertainty of Perception subscale factors. Groups were similar with respect to age, gender, and non-smoker/smoker status (L-HD: seven non-smokers, five smokers; H-HD: six non-smokers, six smokers). All the non-smokers had not smoked a cigarette in the past year, and the smoking characteristics of the smoking in the two groups was similar, with smokers smoking on average for 7.8 years (range 4–12) and smoking a mean of 16.8 cigarettes/day (range 15–20). None of these participants reported a personal or first-degree family psychiatric history, use of medications or a serious medical problem, and none exhibited abnormal results during a physical examination (with electrocardiogram) or with routine laboratory tests (complete blood count, blood chemistry, urine analysis, and urine toxicology for drug use).

The study was approved by the Research Ethics Board of the Royal Ottawa Health Care Group and all participants signed an informed consent prior to study participation.

EXPERIMENTAL DESIGN

The study involved four test sessions couched within a double-blind, placebo-controlled, cross-over design with two parallel groups, L-HD and H-HD. Each session involved the intravenous infusion of ketamine (KI) or a comparable placebo (PI) as well

as oral administration (gum) of nicotine (NG) or a comparable placebo (PG). Two of the four test sessions involved a KI and in the remaining two sessions participants received a PI. Also, in one of the KI and PI sessions, participants were pretreated with NG, and in the other two (KI and PI) sessions they were pretreated with PG. Order of the four treatments (PG–PI, PG–KI, NG–PI, NG–KI) was counterbalanced and sessions were separated by a minimum one-day interval.

SESSION PROCEDURES

In each of the four test sessions, participants arrived at the laboratory (08:00 a.m.) following overnight (beginning 12:00 a.m.) abstinence from food, drugs, alcohol, caffeine, and cigarettes. Smoking abstinence was verified by an expired air carbon monoxide (CO) reading, which was required to be below 5 parts per million (ppm). Following insertion of an antecubital intravenous line, a 45-min adaptation period and EEG electrode placement, participants were administered either NG or PG, and after a 30-min nicotine absorption period, KI or PI was initiated along with the RVIP task and simultaneous ERP recordings for MMN. Vital signs were assessed both before drug treatment and at the end of the session.

DRUG ADMINISTRATION

Nicotine

An oral dose of nicotine was administered in the form of nicotine polacrilex gum, providing delivery primarily *via* buccal absorption (Hukkanen et al., 2005). A 4 mg Nicorette Plus (Hoechst Roussell) gum piece or matching placebo was chewed (with nose plugs, to help reduce any possible sensory impact differences between nicotine and placebo) over a 25-min period according to manufacturer guidelines, with participants using an audiotape to instruct them to bite the gum twice per minute and to *park* the gum (i.e., inside the mouth between teeth and cheeks) after each bite. An additional 5-min absorption period, involving the chewing of a strong mint gum to help disguise any placebo vs. nicotine flavor differences, followed the gum chewing so that peak blood levels from the subsequent infusion of ketamine coincided with slower rising blood nicotine concentrations, which typically exhibit a T_{max} of ∼30-min and are expected to range between 10 and 17 ng/ml (Hukkanen et al., 2005). Plasma levels of nicotine were not assessed. The elimination half-life of plasma nicotine is approximately 120 min.

Ketamine

Human ketamine studies of acute psychoses typically involve systemic infusions of ketamine at dose levels well below the 1–2 mg/kg dose range used in human anesthesia, either by administering a constant dose (0.5 mg/kg) over a ∼60-min period (Krystal et al., 1994), or by administering an initial bolus (Malhotra et al., 1996) or loading dose (Newcomer et al., 1999), both followed by infusion of a maintenance dose of ketamine to achieve steady state ketamine blood levels over a ∼60-min period. As loading doses of 0.27 and 0.08 mg/kg have produced marked and mild psychotomimetic-like reactions, respectively, with no subjective effects or measurable plasma ketamine levels being observed with 0.024 mg/kg (Newcomer et al., 1999), this study utilized an intermediate low loading dose of 0.04 mg/kg, but no maintenance dose, with the aim of avoiding the experience of schizophrenia-like clinical symptoms. *Although there are significant relations between MMN Amplitudes and psychoses-like symptoms induced by ketamine* (Umbricht et al., 2002), *our use of a relatively low dose allows us to study the effects of ketamine independent of psychotic symptoms.* An automated pump apparatus (Imed Gemini PC-1) infused 0.04 mg/kg ketamine hydrochloride or placebo [saline (0.9% sodium chloride) solution] over a 10-min period in order to maximize safety. This dose level, previously used in our laboratory, had been associated with arousal EEG, and cognitive changes in healthy volunteers (Knott et al., 2006, 2011) and produced ketamine blood levels (25–37 mg/ml) that equated with levels seen in a prior study that administered a 0.08 mg/kg dose of ketamine (Newcomer et al., 1999). Although not able to be assessed in all the study participants due to prohibitive costs, gas chromatography (National Medical Services, Philadelphia, PA, USA) carried out on five plasma samples obtained from the PG–KI condition (collected at termination of the infusion) found KI to produce ketamine levels ranging from 25 to 37 ng/ml ($M = 31$ ng/ml). These ketamine levels approximate the average ketamine level reported with the 0.08 mg/kg dose of ketamine (Newcomer et al., 1999).

STUDY ASSESSMENTS

In order to minimize attention to the auditory stimuli eliciting the MMN, these ERPs are typically acquired while participants are engaged in a secondary visual task, which in this case, was the RVIP task.

RVIP paradigm

Employing the original RVIP paradigm of Wesnes and Warburton (1984), participants viewed 50-ms duration single digits (1–9) presented (black on white) in the center of a monitor at a fixed rate of 110 digits/min, the average inter-stimulus interval being 545 ms and ranging between 445 and 645 ms. Button presses were required on the detection of targets, which were defined as the third of three consecutive odd digits. Over the 12-min task, a total of *1320* digits, *including 120 targets* (10 per min) were presented, with each target sequence being separated by a minimum of five digits. Volunteers were instructed to respond (with right index finger) as quickly and accurately as possible and performance measures included: the number of correct responses (expressed as % hits), with correct responses being defined as a button press to the third digit of each target triad within 100–1000 ms post-stimulus onset; the number (%) of incorrect or false alarm (FA) responses, defined as button presses to non-target stimuli, and reaction times (RT) of correct responses. *In addition, signal detection methodology was used to equally weight hits and FAs into a single measure (d'), which is considered to be a purer index of perceptual accuracy/sensitivity* (Macmillan and Creelman, 1997). Response measures were separated into three successive time blocks of 4-min each to assess performance changes with drugs across time.

MMN paradigm

Auditory stimuli eliciting the MMN during ERP recordings consisted of a total of 1200, 80 dB tones (50 ms duration 10 ms

rise/decay time) presented binaurally through headphones (80 dB sound pressure level) with a stimulus onset asynchrony of 545 ms (ranging between 445 and 645 ms) and between individual RVIP stimuli; resulting in no temporal overlap of the two stimulus modalities. Ten percent of the tones (deviants) were 100 Hz and were randomly presented among the standard tones (1000 Hz). Electrical activity was recorded from frontal (F_z), central (C_z), and parietal (P_z) midline scalp sites *using a* linked earlobe *reference. Although choice of reference site may potentially alter outcomes, ketamine-induced reductions in MMN have been observed with a variety of references, including linked ears* (Gunduz-Bruce et al., 2012) *and linked mastoids* (Umbricht et al., 2000). *Recordings were also taken* from orbital sites *and* amplifier bandpass filters and EEG sampling rate set at 0.1–40 Hz and 250 Hz, respectively. ERP processing included epoch segmentation (500 ms, beginning 50 ms pre-stimulus onset), ocular correction, artifacting (eliminating ocular corrected epochs with EEG > 100 μV), baseline correction, and separate averaging of standard and deviant epochs. As with the RVIP data, averages were separated into three equal time blocks, with each block containing 400 stimuli (40 deviants). There were no significant differences between the study conditions in terms of the number of epochs comprising standard and deviant averages and all deviant averages contained a minimum of 30 stimuli. MMNs were derived by digital point-by-point subtraction of standard waveforms from deviant waveforms. *Since out own work found frontal MMN in schizophrenia to be effected by acute nicotine* (Dulude et al., 2010) *and as ketamine-induced reductions in MMN in healthy volunteers was evidenced only at frontal (vs. temporal) sites* (Schmidt et al., 2012a), *the* MMN was measured from F_z, the site of maximal amplitude. Based on grand averages, *MMN amplitude* was defined as the peak negative voltage (relative to pre-stimulus baseline) in an 80–220 ms window. Latency (ms) was measured as the time (from stimulus onset) to reach peak negativity.

STATISTICAL ANALYSIS

Performance measures (hits, FA, d', RT) were subjected to separate split-plot ANOVAs with one between-group factor (HD, two levels) and three within-group factors (gum, two levels; drug, two levels; time block, three levels). Similar ANOVAs were carried out with the ERP measures (MMN amplitude and latency). Greenhouse–Geisser corrections were applied where appropriate to compensate for violations of sphericity assumed with univariate ANOVAs. Significant ($p < 0.05$) main or interaction effects were followed up with Bonferroni adjusted pairwise comparisons.

Exploration of relationships between MMN and sustained attention were examined with Pearson r correlations between MMN amplitude and d′ (collapsed across groups and time blocks) using placebo data (i.e., PG plus PI condition) as well as change score data, derived by subtracting values in the placebo condition (PG–PI) from values derived in each of the three non-placebo sessions.

RESULTS

EFFECTS ON MMN

Robust MMN amplitudes to pitch deviants were shown in each of the four test sessions and in each of the three time blocks within each session. No significant main effects were observed for drug, gum, group, or time block but a significant group × drug × block ($F = 4.05$, d$f = 2, 44$, $p = 0.031$) interaction was demonstrated for amplitudes. Although exhibiting a general amplitude reduction with KI, planned comparisons showed that compared to placebo, KI (vs. PI) significantly reduced MMN only during time block 3 in the H-HD group ($p = 0.038$). In addition, the MMN amplitude of the H-HD group during time block 3 was significantly smaller ($p = 0.05$) than that of the L-HD group during KI (**Figure 1**).

Follow-up analysis of a significant group × drug × gum interaction ($F = 3.945$, d$f = 1, 22$, $p = 0.049$) showed a significant ketamine effect in the H-HD group. More specifically, when administered with PG, KI (vs. PI) significantly ($p = 0.004$) attenuated

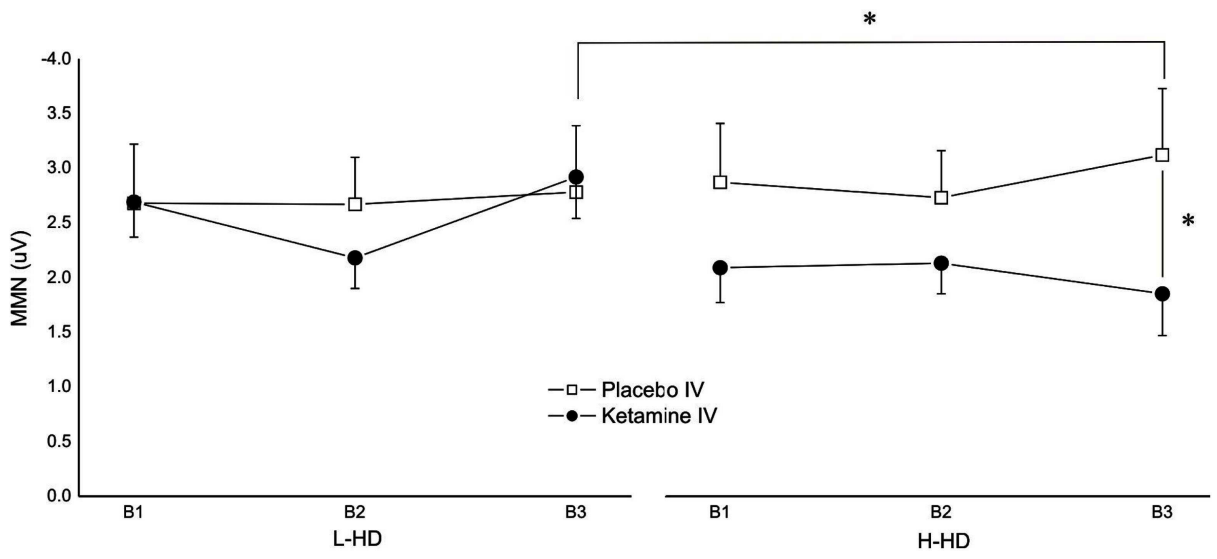

FIGURE 1 | Effects of placebo and ketamine infusion (collapsed across placebo and nicotine gum) on frontal (Fz) mean (± SE) MMN amplitudes of L-HD and H-HD participants in the three time blocks (B1–B3; *$p < 0.05$).

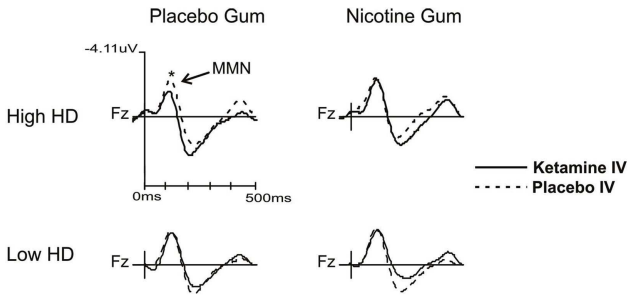

FIGURE 2 | Grand averaged waveforms showing MMN changes (collapsed across time blocks and L-HD and H-HD groups) with ketamine and placebo infusions when combined with nicotine and placebo gum (*$p < 0.05$).

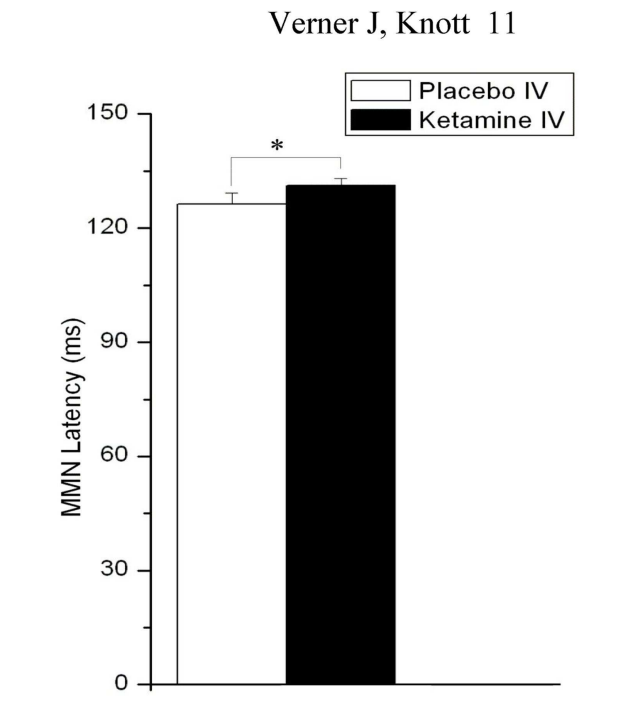

FIGURE 3 | Effects of placebo and ketamine infusion (collapsed across gum conditions, time blocks, and L-HD and H-HD groups) on mean (± SE) frontal (F*z*) MMN latency (*$p < 0.05$).

MMN amplitudes of the H-HD participants (**Figure 2**). Additionally, in this same condition when KI was combined with PG, the H-HD exhibited significantly smaller MMN amplitudes than the L-HD group ($p = 0.004$). In the H-HD group, KI did not attenuate MMN when it was administered with NG (i.e., nicotine blocked KI effects on MMN) and MMN was shown to be significantly smaller when KI was combined with PG compared to when it was combined with NG ($p = 0.007$).

In addition to amplitude, latency of MMN was slightly but significantly affected by drug ($F = 4.38$, d$f = 1, 22$, $p = 0.048$), with KI resulting in a slowing of MMN relative to PI (**Figure 3**).

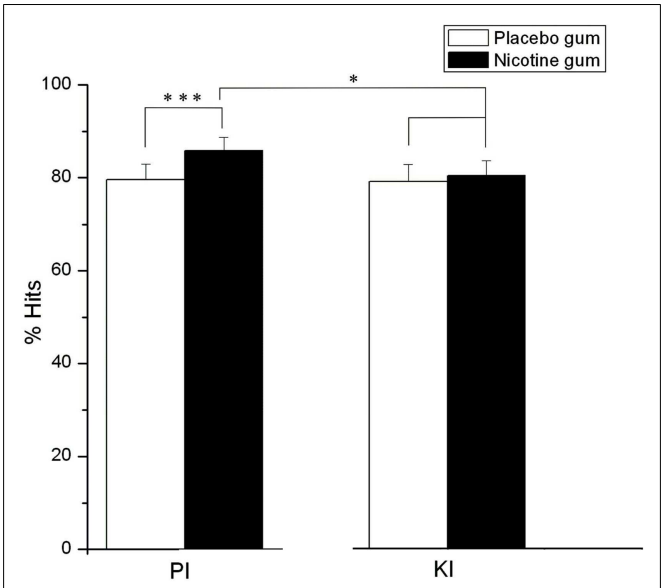

FIGURE 4 | Effects of placebo and nicotine gum (collapsed across time blocks, and L-HD and H-HD groups) on mean (± SE)% hits during placebo (PI) and ketamine (KI) *infusion* (*$p < 0.05$; ***$p < 0.001$).

EFFECTS ON RVIP

Response accuracy as expressed by % hits varied significantly in relation to time block ($F = 5.27$, d$f = 2, 44$, $p = 0.01$), with hits being reduced ($p = 0.01$) in time blocks 2 ($M = 79.94\%$, SE ± 4.98) and 3 ($M = 79.16\%$, SE ± 5.18) compared to time block 1 ($M = 84.38\%$, SE ± 3.76). A significant gum ($F = 11.15$, d$f = 1, 22$, $p = 0.003$) and a gum × drug interaction ($F = 10.05$, d$f = 1, 22$, $p = 0.004$) demonstrated a general enhancing effect of NG (vs. PG) on target detection, which was blocked by KI. Specifically, in the PI conditions, NG significantly elevated % hits relative to PG ($p = 0.0001$) but this gum effect was not observed under KI conditions (**Figure 4**). The blockade of nicotine-induced increases in hit rates was further reflected in the comparison of the two NG conditions, where hits during the combined NG-KI condition were significantly reduced compared to when nicotine was combined with PI ($p = 0.05$) *and when KI was administered alone* ($p < 0.05$).

In a group × gum × block interaction ($F = 3.64$, d$f = 1, 44$, $p = 0.046$), the H-HD group exhibited significantly ($p = 0.04$) reduced % hits than the L-HD group in time block 1 ($p = 0.05$) and time block 2 (**Figure 5**). For the H-HD group, NG (relative to PG) was shown to elevate hit rate in the H-HD during time block 1 ($p = 0.006$) and during time block 2 ($p = 0.001$).

As shown in **Figure 6**, FAs were moderated by a gum × time block interaction ($F = 3.23$, d$f = 2, 44$, $p = 0.032$), with NG acting to prevent the response errors seen in time block with PG ($p = 0.038$).

The analysis of d' yielded a significant gum ($F = 13.63$, d$f = 1, 44$, ($p = 0.001$), drug × gum ($F = 7.06$, d$f = 1, 44$, ($p = 0.014$) and a drug × gum × block × group effects ($F = 3.64$, d$f = 1, 44$, ($p = 0.05$). NG (vs. PG) increased scores ($p = 0.001$) but this was shown only during PI (not KI) and, as displayed in **Table 1**, was

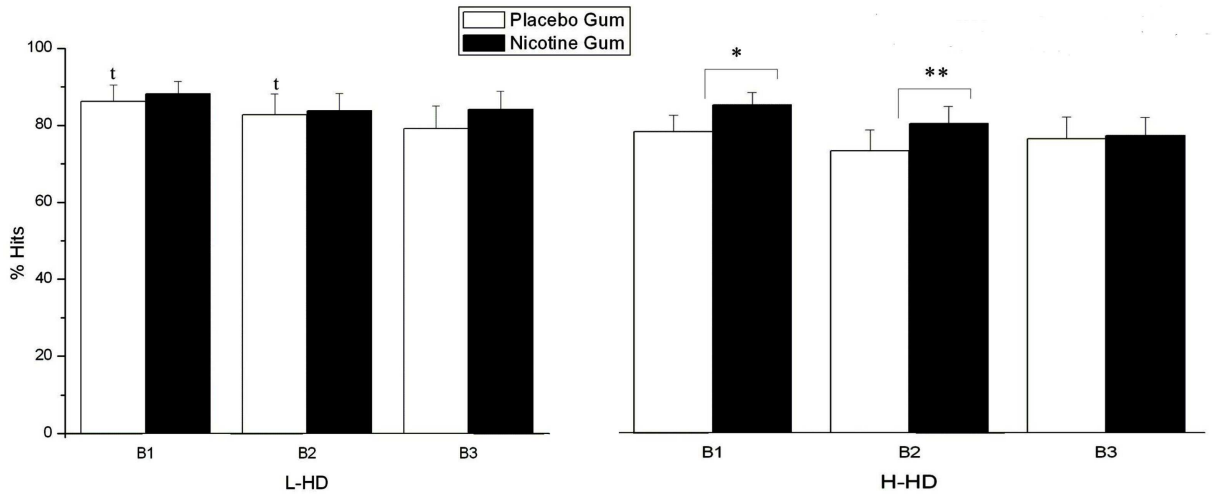

FIGURE 5 | Effects of placebo and nicotine gum (collapsed across placebo and ketamine infusions) on mean (±SE) % hits of L-HD and H-HD groups in the three times blocks (B1–B3). (*$p < 0.006$; **$p < 0.001$; *t*, $p < 0.05$ comparing L-HD and H-HD with placebo gum in B1 and B2).

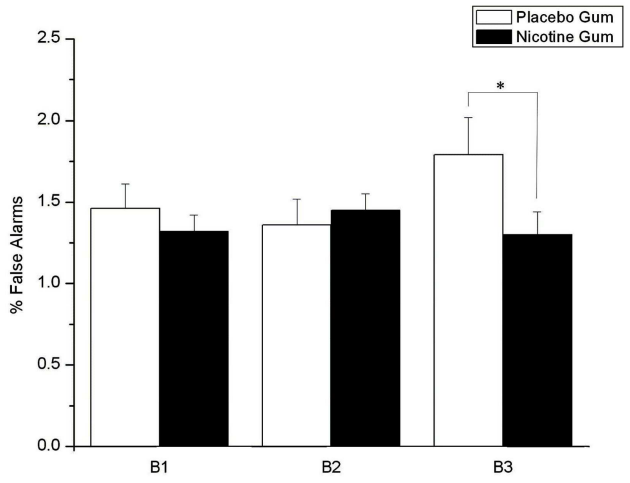

FIGURE 6 | Effects of placebo and nicotine gum (collapsed across placebo and ketamine infusions, and L-HD and H-HD groups) on mean (±SE) % false alarms in the three time blocks (B1–B3; *$p < 0.05$).

limited to the H-HD group during time block 1 ($p = 0.001$) and 2 ($p = 0.009$). During administration of placebo (PG–PI), d' scores of the H-HD group were also significantly lower than those of the L-HD group in time block 1 ($p = 0.05$) and 3 ($p = 0.05$). Also for the H-HD group in the combined Ki-NG condition, d' scores significantly declined from block 1 to block 2 ($p = 0.01$).

Response speed was significantly influenced by gum ($F = 4.77$, $df = 1, 22$, $p = 0.040$), with NG speeding RT relative to PG. In a group × drug interaction ($F = 4.77$, $df = 1.22$, $p = 0.040$) RT of the H-HD group was found to be significantly slower ($p = 0.032$) than the L-HD during administration of PG and PI (**Figure 7**). Although not affecting the H-HD participants, *there was a trend for* KI (vs. PI) *to slow* RT ($p = 0.06$) in the L-HD group, and during the KI condition there were no differences in RT between the L-HD and H-HD groups.

CORRELATIONS

No significant correlations were observed between MMN and d′ in the placebo condition (PG–PI) nor were there any significant correlations between ketamine/nicotine-induced changes in MMN and ketamine/nicotine-induced change in d′.

DISCUSSION

To our knowledge, this is the first study to investigate the moderating role of nicotine on ketamine-induced changes in both sensory and attentional processing, two domains which are highly dysfunctional in schizophrenia and are differentially modulated by the NMDAR antagonist ketamine and the nAChR agonist nicotine. Specifically, the study investigated the hypothesis that MMN – an index of auditory sensory memory that depends on intact NMDAR functioning, would be impaired by ketamine but not when co-administered with nicotine. The results support this hypothesis in that healthy volunteers, assessed to be more prone to experiencing hallucinatory activity (H-HD), evidenced a significantly diminished MMN generation with ketamine alone but not when ketamine was combined with nicotine. During ketamine infusion, MMN amplitudes of H-HD participants were significantly reduced compared to L-HD participants, assessed to be less prone to HD.

Although not observed in one previous study (Oranje et al., 2000), the reduction of MMN with intravenous ketamine is consistent with prior reports of MMN attenuation in healthy volunteers with sub-anesthetic doses of the NMDA antagonist ketamine (Umbricht et al., 2000) and parallels the reduction of MMN in monkeys following infusion of NMDAR antagonists into primary auditory cortex (Javitt et al., 1992, 1994, 1996) as well as the dose-dependent blockade of the MMN recorded from rat auditory cortex after intra-peritoneal injections of the NMDAR

Table 1 | Mean/±SE *d′* scores of L-HD and H-HD groups across treatment conditions and time blocks (B1–B3).

Treatment	L-HD			H-HD		
	B1	B2	B3	B1	B2	B3
PG–PI	3.49/0.18	3.45/0.27	3.39/0.28	3.10/0.18	3.02/0.27	2.95/0.28
PG–KI	3.44/0.23	3.38/0.24	3.04/0.29	3.26/0.23	3.04/0.24	3.38/0.29
NG–PI	3.66/0.19	3.62/0.24	3.49/0.25	3.86/0.19	3.27/0.24	3.44/0.25
NG–KI	3.46/0.21	3.23/0.2	3.44/0.25	3.26/0.21	3.29/0.22	3.05/0.25

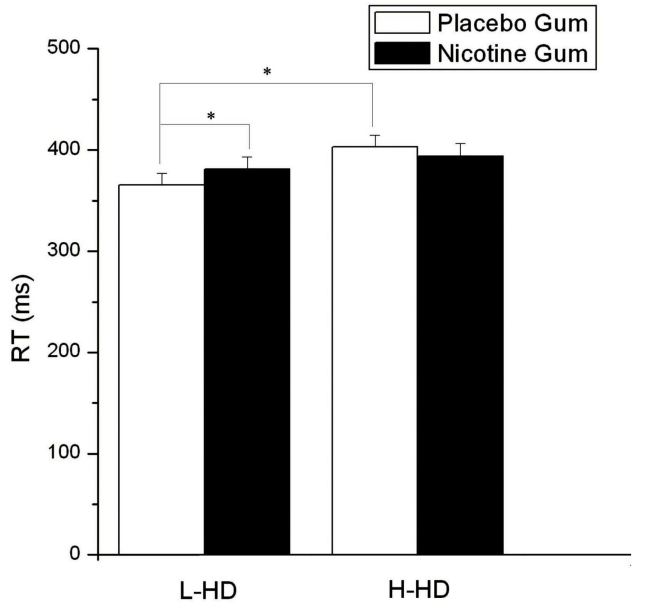

FIGURE 7 | Effects of placebo and nicotine gum (collapsed across placebo and ketamine infusions and the three time blocks) on mean (±SE) reaction time (RT) in L-HD and H-HD groups (*$p < 0.05$).

antagonist, MK-P01, and memantine (Tikhonravov et al., 2008, 2010). Together with the observation of robust reductions of MMN (effect size of 0.99) in schizophrenia patients (Umbricht and Krljes, 2005) and the wealth of evidence suggesting that psychosis is secondary to NMDAR hypofunction with a downstream effect in dopaminergic activity (Fu et al., 2000; Lin et al., 2012), these present findings provide indirect evidence supporting the glutamate/NMDAR hypothesis of schizophrenia and specifically reinforce the implication that deficits in glutamatergic signaling underlie auditory sensory memory impairment characterizing this disorder.

Unlike previous cognitive and MMN studies that purposefully used ketamine doses which were psychotomimetic (i.e., typically involving initial loading dose of ∼0.25 mg/kg followed by a maintenance dose of ∼0.5–0.9 mg/kg/h) and transiently reproduced schizophrenia-like negative and positive symptoms in healthy volunteers (e.g., Newcomer et al., 1999), this present study used a single, low dose bolus infusion (0.04 mg/kg) which had previously been shown to induce only mild subjective arousal and euphoric but not clinically relevant behavioral changes. MMN is indirectly proportional to the prevalence of negative symptoms (Urban et al., 2007) and as such, ketamine-induced behavioral effects with psychotomimetic doses may potentially contribute to cognitive deficits including sensory processing abnormality. However, in healthy controls administered the psychotomimetic psilocybin (a 5-HT2A agonist), a reduction in MMN was not shown despite the experienced marked behavioral changes (Umbricht and Vollenweider, 1999). Viewed in the context of previous reports of greater MMN deficits in patients prone to AH (Fisher et al., 2008, 2011, 2012a,b) and of studies showing the magnitude of MMN deficit in patients to be correlated with clinical ratings of hallucinatory behavior (Youn et al., 2003; see also, Hirayasu et al., 1998 and Thonnesen et al., 2008), our finding of diminished MMN generation with a low sub-psychotomimetic dose of ketamine in H-HD (vs. L-HD) participants suggests that glutamatergic neurotransmission in the auditory cortex of individuals with a trait that predisposes to AH may be more vulnerable to disruption of NDMAR activity.

Keeping in mind that AHs of non-patients may not be similar to those of psychotic or non-psychotic clinical patients (Daalman et al., 2011), hallucinations in general are essentially perceptions that arise through an interaction stemming from neural activations and top-down activity. The basic neural signal contributing to a tonic trait-like vulnerability to experience AHs appears to arise from hyperactivation of functional networks involving the auditory cortex that generate aberrant auditory signals (Kuhn and Gallinat, 2010; Waters et al., 2012). Although AHs were not formally assessed during the testing sessions, it is possible that NMDAR antagonism induced an abnormality in auditory signaling and increased hallucinating activity, which may have impacted MMN generation. In support of this argument, patients in general exhibit reduced sensory-level processing of auditory input (e.g., diminished auditory acuity, elevated thresholds for tone discrimination; Mathew et al., 1993; Holcomb et al., 1995; Strous et al., 1995; Wexler et al., 1998; Rabinowicz et al., 2000); patients with (vs. without) AHs have greater difficulty in sound (speech) discrimination (Hugdahl et al., 2008), and the temporal cortex evidences both high glutamate levels in patients (Marsman et al., 2011) and hypofunctional activation in controls during ketamine infusion (Hugdahl et al., 2008). However, impaired auditory MMN generation during ketamine infusion is unlikely to be related to competing influences resulting from increasing auditory verbal hallucinations as organized AH (i.e., identifiable as speech) as seen in established schizophrenia are rarely observed during a ketamine challenge (Kantrowitz and Javitt, 2010). Also, any evoked auditory disturbances during ketamine infusion resemble the pattern observed in the early course of schizophrenia and as such, altered auditory processing during a ketamine challenge may be

viewed more as a model of prodromal or acute incipient schizophrenia, rather than late, chronic schizophrenia (Kantrowitz and Javitt, 2010).

Nicotine alone did not affect MMN but it prevented the MMN attenuation induced with ketamine. Failure to observe enhanced sensory memory processing with nicotine as shown in earlier reports may be related to methodological differences between studies, including dose and route of nicotine administration, stimulus feature and presentation parameters, ERP recording and processing procedures, and composition of participant samples. However, the ability of nicotine to block the NMDAR antagonist actions of ketamine on auditory sensory memory is consistent with findings that acetylcholine is one of the major modulators of auditory cortical activity (Edeline, 2003; Soto et al., 2006), with both high (α4β2) and low (α7) nicotine affinity nAChRs being densely, and widely expressed in the auditory pathway (Morley and Happe, 2000), particularly where thalamocortical inputs terminate (Metherate, 2004; Bieszczad et al., 2012).

The underlying mechanism of nicotine's protective action is unclear and although it may involve the regulation of sensory processes by tonic release of endogenous acetylcholine at nAChRs, thalamocortical synapses are excitatory and glutamatergic (Kharazia and Weinberg, 1994). Hence, nicotine blockade of ketamine effects may implicate nicotinic regulation of thalamocortical glutamate release by presynaptic (or preterminal) nAChRs, and possibly nicotinic regulation of GABAergic interneurons (Radcliffe et al., 1999; Schilström et al., 2000; Metherate and Hsieh, 2003; Liang et al., 2008; Intskirveli and Metherate, 2012). As an additional or alternative mechanism, nicotinic agonists have also been shown to inhibit NMDAR-mediated cortical currents, possibly by displacing the obligatory NMDAR co-agonist glycine (Flores-Hernandez et al., 2009). Regardless of the mechanism of action, these present study findings of diminished ketamine-induced MMN impairment were observed with a smoking-dose of nicotine, as was the previous finding of nicotine-induced normalization of MMN in schizophrenia patients (Dulude et al., 2010). Together they provide tentative support for the contention that excessive tobacco use in schizophrenia in patients may be an attempt to correct sensory deficiencies related to dysfunctional nAChR and/or NMDAR systems. The increase in MMN latency with NMDAR antagonism, reported previously by other studies using larger ketamine doses (Umbricht et al., 2000; Kreitschmann-Andermahr et al., 2001; Roser et al., 2011), was not moderated by nicotine, suggesting that nicotine's protective actions in the human ketamine model are specific to the strength and not the speed of sensory memory processes.

Performance in the RVIP task was also investigated and was generally found to be enhanced during acute nicotine administration, with both response accuracy *(hit rate and d′)* and speed (RT) being improved relative to placebo. Indexing sustained attention and working memory aspects of cognition, task performance in CPT paradigms such as RVIP has consistently been impaired both in schizophrenia (Hilti et al., 2010; Barch et al., 2012) and tobacco abstaining smokers (Heishman, 1999). Nicotine has been shown to improve CPT efficiency in schizophrenia (Smith et al., 2006; Barr et al., 2008), with attentional improvements in some studies being reported to be selective for non-smoking (vs. smoking) patients (Harris et al., 2004). *For sustained attention measured with RVIP, nicotine improved but did not normalize performance in schizophrenia and it exerted no significant reversal of the impaired frontal-parietal-cingulate-thalamic attention network associated with schizophrenia* (Hong et al., 2011). Although typically viewed as a task of sustained attention, RVIP also involves a significant working memory component and nicotine improvement of RVIP and attention-dependent tasks may be related to its demonstrated ability to activate posterior (parietal) brain areas traditionally associated with visual attention (Lawrence et al., 2002) and/or to increase functional connectivity in frontal, executive-based cortical regions (Jacobsen et al., 2004).

The increased hit rate and d' seen with nicotine (vs. placebo) gum during placebo infusion was absent when nicotine was administered with ketamine. Ionotropic- but not metabotropic-glutamate antagonist treatment also blocked nicotine improved response accuracy in rodent attentional tasks (Quarta et al., 2007; Amitai and Markou, 2009). Together these observations suggest that nAChR modulation of glutamate release underlies nicotine-enhanced target detection and is diminished by NMDAR blockade. However, nAChR and NMDAR ligands appear to overlap in their ligand affinities (Aracava et al., 2005; Plazas et al., 2007) and as anesthetics are potent inhibitors of presynaptic nAChRs, with ketamine being shown to block α4 β2 nAChRs (Irnaten et al., 2002; Tassonyi et al., 2002), it may have attenuated the nicotinic stimulation required for improvements in correct response rates.

Nicotine-facilitated target detection was found to vary between HD groups, with increased d' and hit rates during nicotine (vs. placebo) administration being limited to the higher scoring individuals (H-HD), who also exhibited reduced hit rates and d' compared to L-HD participants in the non-drug condition. Although in schizophrenia hallucinators, the reduced activation shown in primary auditory cortex during auditory target detection was not seen in visual cortex (Ford et al., 2009), recent neurophysiological evidence has pointed to early visual processing deficits in patients who tend to hallucinate (Kayser et al., 2012). Perhaps extending to healthy controls with a hallucinating propensity, these findings in patient hallucinators suggested a broader early perceptual deficit that extends beyond the auditory modality. The performance improving effect of nicotine on RVIP in H-HD is consistent with previous ERP research showing preferential enhancement in visual (vs. auditory) processing (e.g., increased amplitude of the N100 and P300 ERPs) with smoking/nicotine (Knott, 1989; Knott et al., 1995, 1999; Pritchard et al., 2004) and it also parallels reports showing nicotine – induced cognitive facilitation to be more prevalent in poorer performing, cognitively deficient populations (Newhouse et al., 2004).

The H-HD group were also slower in responding to RVIP targets than the L-HD group but ketamine, not nicotine, influenced response speed only in the L-HD participants, increasing RT relative to placebo. Together with reduced target detection rates, response slowing in non-patient hallucinators support and extend the contention applied to schizophrenia voice-hearers (Ford et al., 2009), that their auditory cortex may be "turned on" and tonically "tuned in" to internal acoustic information at the cost of processing not only external sounds but on the basis of these findings, external visual input as well. Perhaps reflecting a "floor" effect that prevents further slowing of RT, ketamine-induced slowing was not seen in H-HD and its appearance in L-HD indicates that,

as shown with nicotine's *attentional* enhancing properties, the *RVIP* impairing actions of NMDAR blockade in healthy volunteers are baseline dependent. *MMN failed to show any relationship to d′ in the placebo or active conditions. In schizophrenia, MMN has been found to be related and contribute to higher-order cognitive impairments as well as deficits in social cognition* (Javitt et al., 1995; Baldeweg et al., 2004; Sehatpour et al., 2010; Wynn et al., 2010). *Unlike our present findings, modeling study suggested ketamine reductions in MMN in healthy volunteers to be mediated by changes in short-term plasticity (of "forward" inter-regional connections) of the auditory hierarchy, which significantly correlated with ratings of ketamine-induced impairments in cognition and control (Schmidt et al., 2012b). Although RVIP may be an exception, re-examination of this task in larger sample of healthy volunteers and patients and with larger doses of nicotine and ketamine may show unique relationships between MMN and sustained attention that are moderated by SZ pathology and/or nicotinic/glutamatergic activity.*

LIMITATIONS

Methodological weakness in the present study may have moderated the findings and limited the conclusions and implications of this research. First, both nicotine and ketamine were administered as single doses and accordingly, it is not know if the observed results with each drug and their combination are dose-dependent. Second, ketamine was infused as a low bolus dose and the related cognitive findings may be distinct from those observed with the majority of human ketamine studies that have assessed cognition with sustained psychotogenic doses of this drug. Also, blood levels of the two drugs were not assayed to determine bioavailability and the cognitive effects of nicotine administered via slow buccal absorption may not mirror the effects of acute smoking, which delivers rapid nicotine boli to the brain. *Performance* assessments were also limited and did not encompass the range of information processing deficits that characterize schizophrenia, each of which are valid targets for novel nicotinic and glutamatergic therapies. Finally, the L-HD and H-HD study samples were relatively small *and* were drawn from a healthy population and although there are advantages to this approach, similar challenge studies are required with healthy surrogate populations (e.g., schizotypal personality disorder, unaffected first-degree relatives of patients) with genetic links to schizophrenia.

CONCLUSION

This study produced novel findings which underscore the potential role of nAChR and NMDAR systems and their interplay in the etiology of core neurocognitive deficits characterizing schizophrenia. The disturbance in sensory and attentional processing evident with a sub-psychotomimetic dose of ketamine reflects the widespread distribution of brain NMDARs and their sensitivity to minimal perturbations in glutamate signaling. Nicotine exhibited strong *attentional enhancing* properties, which in part were dependent on glutamate neurotransmission and were also evident by its ability to counter *impaired deviance detection* induced with NMDAR blockade. That these effects were moderated by a hallucinatory trait in a healthy population suggests that nicotine's sensory-*attentional* properties and its modulatory effects on NMDAR systems are relevant to neurocognitive deficits in schizophrenia and may be specific to patient subtypes.

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Cotinine: beyond that expected, more than a biomarker of tobacco consumption

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A greater incidence of tobacco consumption occurs among individuals with psychiatric conditions including post-traumatic stress disorder (PTSD), bipolar disorder, major depression, and schizophrenia, compared with the general population. Even when still controversial, it has been postulated that smoking is a form of self-medication that reduces psychiatric symptoms among individuals with these disorders. To better understand the component(s) of tobacco-inducing smoking behavior, greater attention has been directed toward nicotine. However, in recent years, new evidence has shown that cotinine, the main metabolite of nicotine, exhibits beneficial effects over psychiatric symptoms and may therefore promote smoking within this population. Some of the behavioral effects of cotinine compared to nicotine are discussed here. Cotinine, which accumulates in the body as a result of tobacco exposure, crosses the blood-brain barrier and has different pharmacological properties compared with nicotine. Cotinine has a longer plasma half-life than nicotine and showed no addictive or cardiovascular effects in humans. In addition, at the preclinical level, cotinine facilitated the extinction of fear memory and anxiety after fear conditioning, improved working memory in a mouse model of Alzheimer's disease (AD) and in a monkey model of schizophrenia. Altogether, the new evidence suggests that the pharmacological and behavioral effects of cotinine may play a key role in promoting tobacco smoking in individuals that suffer from psychiatric conditions and represents a new potential therapeutic agent against psychiatric conditions such as AD and PTSD.

Keywords: cotinine, tobacco, fear, memory, nicotine

INTRODUCTION

The use of tobacco dates back as early as 5,000–3,000 BC, when tobacco plants were first cultivated in South America (Gately, 2003). Initially, tobacco was regarded has a medicinal plant to be used for medical purposes such as, a painkiller for earaches and toothaches (Balls, 1962). However, later studies clearly established the deleterious effects of tobacco smoking on health (Peto et al., 1996). As a result, the progressive establishment of more restrictive anti-tobacco laws has discouraged tobacco smoking worldwide. These new restrictions and public health campaigns have dramatically decreased tobacco use. However, there is a high rate of tobacco consumption among individuals that suffer from mental disorders such as, major depression disorder (MDD), schizophrenia, and post-traumatic stress disorder (PTSD; Leonard et al., 2001; Weaver and Etzel, 2003; Thorndike et al., 2006; Buggia-Prevot et al., 2008; Aubin et al., 2012). The idea that tobacco consumption in these populations is a form of self-medication is controversial and some evidence suggests that smoking is associated with poorer mental health outcomes in some mental disorders such as, bipolar and schizoaffective disorder (Dodd et al., 2010). The desire to identify the component of tobacco that may explain this correlation has encouraged the study of the mental effect(s) of nicotine [3-(1-methyl-2-pyrrolidinyl) pyridine], an alkaloid that is present in tobacco leaves, over the psychiatric symptoms. Nicotine treatment had been shown to improve cognitive function including attention, concentration, executive function, and learning and memory (Elzinga and Bremner, 2002; Horner and Hamner, 2002; Bowie and Harvey, 2005; Gray and Roth, 2007; Tapia et al., 2007; Burriss et al., 2008; Luck and Gold, 2008; Terry et al., 2008; Hinkelmann et al., 2009; Johnsen and Asbjornsen, 2009; Veltmeyer et al., 2009; Baune et al., 2010). However, nicotine's undesirable cardiovascular and addictive side-effects have limited its therapeutic use (Karaconji, 2005). Recently, new studies have shown that the main metabolite of nicotine, an alkaloid named cotinine [(5*S*)-1-methyl-5-(3-pyridyl)-pyrrolidin-2-one], has beneficial therapeutic properties, while not having nicotine's negative side-effects. In preclinical studies, cotinine has shown to improve reference and working memories, attention, and the extinction of fear memory, as well as to reduce both the startle response and anxiety in animal models of aging, Alzheimer's disease (AD), PTSD, and schizophrenia (**Figure 1**). Here the psychopharmacology and the potential therapeutic use of cotinine are discussed.

DISCUSSION

COTININE PHARMACOKINETIC

Cotinine is an alkaloid found in tobacco leaves and the main metabolite of nicotine. The active form of cotinine, the isomer

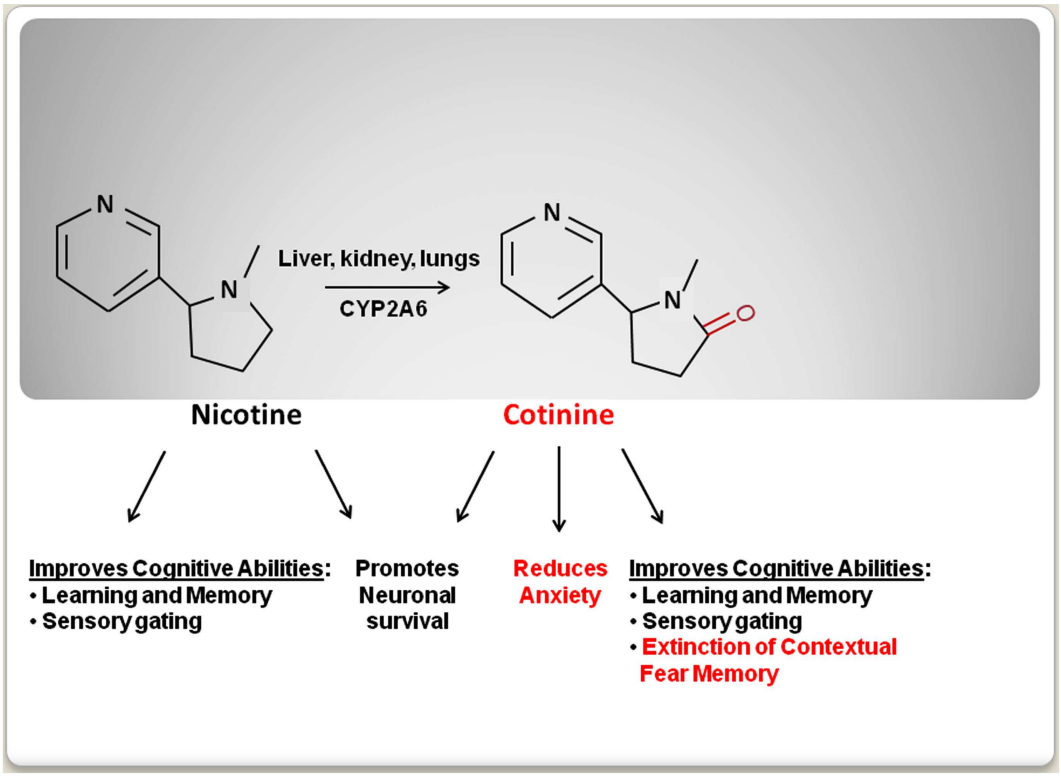

FIGURE 1 | A schematic comparison of the behavioral effects of nicotine and cotinine.

S(-)-cotinine accumulates in the body after tobacco consumption. The pharmacokinetic profiles of cotinine administered orally or intravenously have been investigated in humans. These reports show that cotinine is well-absorbed orally (De Schepper et al., 1987), has a long plasma half-life (19–24 h; Benowitz et al., 1983; Benowitz and Sharp, 1989) and crosses the blood-brain barrier (Riah et al., 1998). Approximately 80–85% of nicotine is metabolized in the liver and converted into cotinine by enzymes such as cytochrome P450 2A6 (CYP2A6; Lewis et al., 1999) and cytochrome P4502A5 (CYP2A5) in human and mouse, respectively (Donato et al., 2000; Visoni et al., 2008).

In humans, cotinine is excreted in the urine, mainly as trans-3′-hydroxycotinine (90% of cotinine) and glucuronide (Caldwell et al., 1992; Ghosheh and Hawes, 2002; Kuehl and Murphy, 2003).

The expression of different variants of the CYP2A6 gene, coding for enzymes with different catalytic activities, influences the level of cotinine achieved in the body after nicotine consumption and, seems to influence nicotine addiction (Malaiyandi et al., 2006; Strasser et al., 2007). For example, individuals that express a catalytically deficient form of CYP2A6 (i.e., CYP2A6*4) showed a lower rate of cigarette consumption compared with individuals that express the normal allele (Yamanaka et al., 2004). The alleles that express the polymorphic forms of the CYP2A6 gene, with low or high enzymatic activity, are distinctly represented in diverse ethnic groups (Bramer and Kallungal, 2003; Nakajima et al., 2006). The distinct expression of the different forms of CYP2A6 may explain the varying metabolism of cotinine observed in individuals of diverse ethnic backgrounds (Nakajima et al., 2006). Therefore cotinine metabolism and, consequently, smoking behavior can be influenced by ethnicity. Furthermore, other factors such as the consumption of a specific food can also affect cotinine metabolism (Nakajima et al., 2006). For example, some components of grapefruit juice inhibit the activity of CYP2A6 and, consequently, cotinine synthesis (Tassaneeyakul et al., 2000; Hukkanen et al., 2006).

COTININE PHARMACODYNAMIC EFFECTS

Early studies of cotinine effects in humans showed that cotinine has a good safety profile (Borzelleca et al., 1962; Bowman and Mc, 1962). One of these seminal studies demonstrated that daily doses of cotinine of up to 1,800 mg for a period of 4 days did not induce deleterious side-effects in humans (Bowman and Mc, 1962). Another clinical study investigating the psychogenic effects of cotinine showed that when administered intravenously to abstinent smokers, this compound reduced the self-reported irritability and tobacco cravings experienced by the participants (Benowitz et al., 1983). A follow-up phase II clinical study investigated the effects of cotinine on smoking cessation in an inpatient, 10-day study in abstinent cigarette smokers (Hatsukami et al., 1997). This study showed that oral cotinine treatment of up to 160 mg/day had no addictive, cardiovascular (e.g., heart rate and blood pressure), or behavioral effects in individuals between 21 and 42 years of age (Hatsukami et al., 1997). A follow-up study from the same

research group also found that cotinine at the doses studied did not help with tobacco cessation and antagonized the reduction of the withdrawal symptoms induced by a nicotine patch (Hatsukami et al., 1998). These results confirmed that orally administered cotinine exhibits behavioral effects in humans, likely by modulating the nAChR sensitivity to agonists; however, this concept requires further experimental validation.

Cotinine is a cognitive enhancer in a mouse model of Alzheimer's Disease

Cotinine has been shown to prevent working and reference memory loss in a mouse model of AD (Echeverria et al., 2011). In this study, the ability of cotinine to prevent plaque development and memory loss in the Tg6799 mice (Ohno et al., 2006) was investigated. Two-month-old Tg and same age non-Tg (NT) mice were orally treated with vehicle (PBS) or 2.5 mg/kg of cotinine for 4.5 months via gavage and tested for working memory using the circular platform, the Radial Arm Water maze (RAWM), and cognitive interference tests. These behavioral study showed that cotinine prevented memory loss in Tg6799 mice and significantly decreased plaques burden in the cortex of the Tg6799 mice. Cotinine treatment also reduced the number and size of the amyloid plaques. The decrease in plaque load also correlated with a decrease in the levels of insoluble Aβ in the cortex of the cotinine-treated Tg mice when compared with the vehicle-treated Tg mice (Echeverria et al., 2011). This evidence suggest that cotinine may be a new therapeutic agent against this devastating condition (Echeverria and Zeitlin, 2012).

Cotinine reduced anxiety and fear in a mouse model of PTSD – like symptoms

Post-traumatic stress disorder is an anxiety disorder that appears after exposure to life-threatening events (Martenyi et al., 2007). PTSD is characterized by symptoms such as anxiety, fear, working memory impairment, hyperarousal, emotional numbing, and sleep disorders. These symptoms have been associated with a permanent alteration of the physiological response to stress induced by over-activation of the hypothalamus-pituitary-adrenal axis (Yehuda et al., 1991). These changes are generally accompanied by dysregulation of several neurotransmitter systems including the serotoninergic, dopaminergic, noradrenergic, and cholinergic systems. The hyperarousal and aggressive behavior observed in patients with PTSD are caused, at least in part, by a decrease in serotonin neurotransmission (Nutt, 2000). Consistent with this idea, serotonin reuptake inhibitors (SSRIs) such as paroxetine and sertraline are currently used in the treatment of PTSD (Brady et al., 2000; Davidson et al., 2001; Brady and Clary, 2003; Corchs et al., 2009; Stein et al., 2009). Because serotonin promotes acetylcholine release, and acetylcholine signaling positively affects attention and memory, an increase of serotonin levels in the brain, may simultaneously improve mood, reduce aggressiveness (Buhot et al., 2000), and positively affect cognitive abilities. Although SSRIs are useful in the treatment of PTSD (Cohen et al., 2000), they are effective only in a small percentage of patients (approximately 30%; Veltmeyer et al., 2009).

Post-traumatic stress disorder patients are commonly heavy smokers, and an association between tobacco dependence and PTSD has previously been established (Hapke et al., 2005). In the search of new drugs that could diminish fear and anxiety and enhance the extinction of fear memories, rodent models of fear conditioning (FC) has been extensively used. These animal models have permitted to characterize the effect of drugs, including cotinine, over anxiety, fear, and contextual memory in anxiety disorders. FC is a broadly used model of associative memory that involves the pairing of neutral conditioning stimuli (CS; sound and context) with an aversive unconditioned stimulus (US; electric shock). After conditioning, the presentation of the CS alone elicits both a conditioned fear response (freezing behavior) and anxiety in the animal. Experimentally, the extinction of contextual fear (FE) after FC is expressed as a progressive decrease in fear responses attained after repetitive exposures to the CS (context) in the absence of the pairing with an aversive US (Steckler and Risbrough, 2012).

A devastating symptom in PTSD is the failure to extinguish traumatic memories that force the patient to continue re-experiencing the trauma (Izquierdo et al., 2004). Thus, the identification of drugs that would enable the extinction of fear memories would be an essential therapeutic goal.

One recent study investigated the effect of pre- and post-treatment with cotinine over the stability of contextual fear memory after repetitive or single re-exposure to the CS (Zeitlin et al., 2012). Male adult mice were pre-treated or post-treated with cotinine before or after FC, respectively, and tested for fear responses and anxiety after being exposed to the CS (context). These studies showed that cotinine accelerated the extinction and reduced the stability of the contextual fear memory. FE is considered a "new learning" process that involves the acquisition of inhibitory memories that compete with the original fear memory consolidation. For this reason, it has been proposed that cognitive enhancers may have a positive effect over the extinction of fear memories. Thus the improvement of cognitive abilities induced by cotinine may be relevant for its effects on FE. Thus, cotinine may promote the extinction of contextual fear memory by stimulating the acquisition of new inhibitory memories.

In addition, because cotinine reduces anxiety and helps to extinguish fear memory, it is feasible that cotinine may also be useful, alone or in a combined treatment, with psychotherapy to reduce both non-cognitive and cognitive symptoms of PTSD. However, these ideas need to be tested in placebo-controlled clinical trials.

Cell signaling changes associated with fear extinction triggered by cotinine

This enhancement of contextual FE in mice subjected to FC described above, correlated with an increase in the extracellular signal-regulated kinases (ERK)1/2 (ERKs) activity in the hippocampus (Chen et al., 2005; Fischer et al., 2007). This increase has been regarded as a key molecular event during the fear extinction process because this brain region is involved in contextual FE (Bouton et al., 2006; Quirk and Mueller, 2008) and also because that ERK1/2 inhibition immediately after memory retrieval prevented the extinction of contextual memory (Chen et al., 2005). Consistent with these findings, the enhancement of FE induced by cotinine treatment in the C57BL/6, mice positively correlated

with an increase in the levels of the active form of ERK1/2 (phospho-ERKs) in the hippocampus.

In addition this study showed that cotinine also reduced anxiety in the conditioned mice. Since it has been demonstrated that cotinine inhibited serotonin reuptake and increased its spontaneous release in rat brains (Fuxe et al., 1979), an increase in serotonin in the brain induced by cotinine may explain the reduction in anxiety after FC in the cotinine-treated mice. A similar effect may also explain the common perception of a "calming effect" experienced by tobacco smokers.

COTININE, IS A MEMORY ENHANCER IN ANIMAL MODELS OF SCHIZOPHRENIA-LIKE SYMPTOMS

Effect of smoking on psychiatric symptoms in schizophrenia

Schizophrenia is a mental disorder characterized by cognitive deficits and both positive and negative symptoms (Van Snellenberg, 2009). The positive symptoms include delusions, hallucinations, and difficulty in thought organization and oral expression. The negative symptoms include deficits in attention and motivation, social withdrawal, and emotional numbness (Van Snellenberg, 2009). Although the causes of schizophrenia are not well understood, abnormalities in the activity of several neurotransmitters such as, dopamine, acetylcholine, gamma-aminobutyric acid (GABA), and glutamate have been proposed (Lang et al., 2007). Since the development of clozapine, current drugs used to treat schizophrenia are mostly antagonists of the serotoninergic/dopaminergic signaling systems. These drugs have been effective in the management of positive psychotic symptoms; however, they do not effectively target the negative symptoms and cognitive deficits observed in schizophrenic patients. Thus, the identification of new drugs are needed to adequately treat these symptoms (Biedermann and Fleischhacker, 2011; Karam et al., 2012).

Patients with schizophrenia may often die prematurely due to health risk factors commonly associated with this condition including obesity and smoking behavior (Sagud et al., 2009; van Os and Kapur, 2009; von Hausswolff-Juhlin et al., 2009; Kelly et al., 2011).

A percentage of patients report, that smoking helps in decreasing psychiatric symptoms (Glynn and Sussman, 1990) which, become worse during tobacco withdrawal (Dalack and Meador-Woodruff, 1996). There are few and incomplete clinical data supporting the therapeutic effects of cotinine in psychiatric conditions, however, in one clinical study it was found that smoking high-nicotine cigarettes, compared to smoking de-nicotinized cigarettes, reduced negative symptoms without affecting positive symptoms (Smith et al., 2002). This effect is considered to be the result of the increase of dopamine levels in the nucleus accumbens and prefrontal cortex induced by nicotine (Corrigall and Coen, 1991). This increase in dopamine may stimulate active coping, improve attention, environmental engagement, and emotional responding, that are commonly absent in schizophrenic patients with prominent negative symptoms.

Cognitive impairment is one of the main challenges experienced by these patients. Growing evidence suggests that a deficit in cholinergic neurotransmission plays an important role in mediating cognitive deficits (Araki et al., 2002; Levin, 2002; Money et al., 2012). This evidence suggests that activation of the nicotinic receptors can be useful in treating some symptoms of schizophrenia particularly the cognitive deficits (Taly et al., 2009; Toyohara and Hashimoto, 2010).

The nicotinic receptors as targets of cotinine effects in schizophrenia

The nicotinic receptors are involved in mediating attention, sensory gating, and learning and memory (AhnAllen, 2012; Yakel, 2012). The most broadly expressed nicotinic receptor (90%) is formed by α4 and β2 subunits and binds nicotine with high affinity while, not binding α-bungarotoxin (McGehee and Role, 1995); this receptor is highly represented in the striatum and substantia nigra and, poorly expressed in the neocortex and hippocampus (Rubboli et al., 1994b).

No less relevant, although less expressed, is the α7 nicotinic acetylcholine receptor (α7 nAChR), a low affinity receptor for nicotine that is highly expressed in the midbrain, neocortex, thalamus, and hippocampus, with lower levels in the striatum (Sugaya et al., 1990; Freedman et al., 1993; Rubboli et al., 1994a,b). This receptor that has high affinity for α-bungarotoxin (McGehee and Role, 1995) and, plays a key role mediating several cognitive functions including attention, memory, executive function, and sensory gating (Woodruff-Pak and Gould, 2002; Leiser et al., 2009; AhnAllen, 2012).

A cholinergic deficit has been proposed as a factor leading to cognitive impairment in schizophrenia. Numerous studies have shown that expression of the α7 nAChR is decreased in the brains of patients with schizophrenia when compared to healthy controls (Breese et al., 2000; Leonard et al., 2000). This idea is supported by clinical evidence showing that the acetylcholinesterase inhibitor, galantamine, which increases the synaptic levels of acetylcholine, improved the cognitive abilities in patients with schizophrenia (Ago, 2010; Ago et al., 2011).

The high rate of smoking in individuals with schizophrenia, although not a evidence of causation, has permitted speculation of the beneficial effect of a tobacco-derivative, but not smoking itself (Dalack and Meador-Woodruff, 1999) on some schizophrenia symptoms such as working memory impairment (Leonard et al., 1998, 2001). For example, evidence has been reported suggesting that α7 nAChR expression is significantly lower in schizophrenic non-smokers than in control non-smokers (Mexal et al., 2010).

In a rat model of Schizophrenia-like symptoms, the administration of nicotine attenuated the working memory deficits induced by the dopamine antagonist dizocilpine (Levin et al., 1998; Ciamei et al., 2001). In patients with schizophrenia, nicotine administration via a nasal spray has shown to ameliorate deficits in working memory, attention, and anxiety (Smith et al., 2006; Buckley et al., 2007). In addition, other clinical studies have shown that chronic treatment with nicotine improved the cognitive abilities in schizophrenic patients treated with antipsychotics (McEvoy et al., 1999; Freedman et al., 2008).

Several modulators of nAChRs have been tested against schizophrenia. For example, a partial agonist of the α7 nAChR, DMXB-A (GTS-21) was investigated in a phase 2 clinical study investigating

the therapeutic effects of this drug over the symptoms of schizophrenia. Although GTS-21 improved some negative symptoms, it did not ameliorate the cognitive deficits observed in schizophrenic patients (Olincy and Stevens, 2007; Freedman et al., 2008).

Despite cotinine acting as a weak agonist (100 times less potent than nicotine) of the nAChRs, in rats it reversed the cognitive deficits induced by antagonism of the glutamate receptor, *N*-methyl-D-aspartate receptor (NMDAR), with ketamine. In this rodent model cotinine improved sustained attention and attenuated behavioral alterations induced by ketamine (Terry et al., 2012).

This pro-cognitive effect is not limited to rodent models of this disorder as cotinine, at similar doses, also improved performance accuracy on the delayed matching to sample (DMTS) task in aged rhesus monkeys. The DMTS task assesses recognition memory for novel non-verbal patterns, and tests short-term visual memory. This test has 19 outcome measures, including latency to response, the number of correct choices selected, and the probability of an error after a correct or incorrect response. In this task, cotinine-treated monkeys performed significantly better than vehicle-treated controls (Terry et al., 2005), indicating that cotinine improved visual short-term memory. These results also suggest that cotinine is a general cognitive enhancer that is useful in different species and types of cognitive abilities.

But the positive effects of cotinine are not limited to cognitive abilities. A recent study tested the effect of acute subcutaneous (0.03–10 mg/kg) and chronic oral administration of cotinine on sustained attention and behavioral alterations in rats induced by the glutamate (NMDA) antagonist MK-801 (Terry et al., 2012). The effects of cotinine were assessed in a five-choice serial reaction timed task (5CSRTT), a test broadly used to measure visual attention and impulsivity in rats. The 5CSRTT is implemented in a specially designed operant chamber with multiple response locations ("nine-hole box") using food reinforcers to maintain performance on baseline sessions (about 100 trials). The test gives the rat a brief 0.5 s visual stimulus, measuring the time, and accuracy of the animal's reactions, as well as the errors made by the rat. The 5CSRTT is used for measuring various aspects of attentional control over performance with its main measures of accuracy, premature responding, correct response latencies, and latency to collect earned food pellets (Robbins, 2002; Robinson et al., 2009).

The results showed that acute treatment with cotinine diminished MK-801-induced impairments in accuracy and elevations in timeout responses while increasing the number of completed trials. Furthermore, chronic treatment with cotinine induced similar beneficial effects even when the difficulties of the task were increased. The authors concluded that cotinine was useful in improving sustained attention and, in decreasing the impulsive and compulsive behaviors that were related to the postulated glutamate receptor signaling dysfunction in schizophrenia (Terry et al., 2012).

Effect of cotinine on sensory gating in animal models of schizophrenia

A deficit in sensory gating is also a characteristic of schizophrenic patients as well as patients with other psychiatric conditions such as PTSD, borderline personality disorder, and bipolar disorder (Braff et al., 1992; Swerdlow et al., 2006). Individuals with this deficit cannot filter out irrelevant stimuli, as their brains are unable to inhibit its responsiveness to similar and repeated stimuli. The activation of cholinergic nicotinic receptors in the hippocampus is one of the main molecular mechanisms that control this inhibitory gating. Because the α7 nAChR is required for supporting sensory gating abilities (Freedman et al., 1994), a cognitive ability that is reduced in the patients with schizophrenia, this receptor is considered a potential therapeutic target for diminishing sensory gating deficit in this disorder (Olincy and Stevens, 2007).

The NMDAR antagonist, dizocilpine, or the dopamine agonist, apomorphine are used in rodents to induce memory and sensory gating deficits resembling those observed in persons with schizophrenia (Seillier and Giuffrida, 2009). For example, apomorphine is used to induce in rodents the deficit in prepulse inhibition (PPI) observed in schizophrenia. PPI is the decrease in the startle response to an auditory stimulus after repetitive exposure to the stimulus and a measure of sensory gating. The sensory gating deficit is expressed as an increase in the startle response (Geyer, 2006). To assess the effect of drugs on PPI animals are treated with vehicle, or the study drug, and are exposed to a weak acoustic stimulus (the prepulse); then, changes in the reflexive flinching response (startle) displayed as a result of a second stimulus of higher intensity (the pulse) is measured. Effective antipsychotic agents prevent the inhibition of PPI induced by psychogenic compounds such as apomorphine and dizolcipine. Previous studies have shown that nicotine blocks the apomorphine-induced disruption of the PPI of the acoustic startle in rats (Suemaru et al., 2004).

In fact, several studies have reported a positive effect of smoking on sensory gating and cognitive function in schizophrenia. In one study, the relationship between sensory gating and smoking levels was assessed by investigating the correlation between PPI and smoking behavior in schizophrenic and control patients (Rabin et al., 2009). The authors found that schizophrenic non-smokers have poorer PPI performances than schizophrenic smokers, whose levels did not differ from non-schizophrenic controls.

In addition to the observed effects of nicotine on sensory gating, other studies have demonstrated that nicotine also improved other schizophrenic symptoms (Lyon, 1999). These results support the view that smoking is a form of self-medication to alleviate psychotic symptoms and the unpleasant side-effects of antipsychotics (Matthews et al., 2011). Interestingly, cotinine, the long-lived metabolite of nicotine, also ameliorated the apomorphine-induced deficits in PPI of the acoustic startle response in rats (Risner et al., 1985; Terry et al., 2005). This result suggested that cotinine may have antipsychotic effects and underlie the beneficial effects of nicotine on attention and sensory gating, in addition to its predicted positive effect over working memory. In addition, as mentioned before, cotinine facilitates fear extinction a measure of executive function which has been found also impaired in people suffering from schizophrenia (Holt et al., 2009). Unfortunately, no clinical studies have been performed to investigate the effect of cotinine over these symptoms in schizophrenia.

Table 1 | Potencies and affinities of cotinine and nicotine to different subtypes of nAChRs in the rat brain and values of acute toxicity in mice.

	*I/IC_{50} [^{3}H] nicotine	I/IC_{50} ^{125}I-labeled α-bungarotoxin	Male		Female	
			LD_{50}	LD_{90}	LD_{50}	LD_{90}
Nicotine	$100/(2 \pm 0.1) \times 10^{-7}$	$100/(1 \pm 0.3) \times 10^{-5}$	31 ± 4	43 ± 6	37 ± 6	51 ± 9
Cotinine	$100/(2 \pm 0.2) \times 10^{-3}$	$100/(1 \pm 0.2) \times 10^{-3}$	2 ± 0.1	4 ± 0.1	3 ± 0.1	4 ± 0.1

**I/IC50, percentage maximum inhibition/concentration of drug that inhibits 50% radioligand binding (in M). Values of IC50, mean ± SD. LD, lethal dose with nicotine attributed 100% toxicity (Riah et al., 1999).*

MOLECULAR MECHANISMS POTENTIALLY MEDIATING THE BENEFICIAL EFFECTS OF COTININE ON COGNITION

Thus far, the molecular mechanisms of cotinine action have been elusive. A previous report suggested that cotinine binds to an unknown type of receptor (Riah et al., 2000). Unfortunately, no follow-up studies pursued the characterization of this "cotinine receptor". Other study based in the fact that cotinine increased serotonin levels in the rat brain, investigated whether granisetron, a 5HT(3) receptor antagonist, could enhance the efficacy of the nicotine patch. The results indicated that 5HT(3) antagonism was an unlikely mechanism of cotinine's actions (Hatsukami et al., 2003). On the other hand, pharmacological evidence using ^{125}I-labeled α-bungarotoxin and [^{3}H] nicotine to differentiate subtypes of nAChRs affinities show that cotinine is a weak agonist of the α7 nAChRs and nicotine has more than 100 times higher affinity than cotinine for agonist binding sites of both subtypes of receptors. In addition the toxicological analysis using acute intraperitoneal injections of drugs in saline, showed that cotinine has in comparison to nicotine less than 1.5% toxicity (**Table 1**; Riah et al., 1999).

It has been suggested that α7 nAChRs is not the main target of cotinine (Riah et al., 1999) and other type of receptors for cotinine have been suggested (Riah et al., 2000). Also, using [^{3}H]dopamine release assays and ligand-binding autoradiography in monkey striatum, it was concluded that cotinine functionally interacts with both α4β2 and α3α6β2 nAChR subtypes in the caudate with a IC_{50} for the inhibition of specific agonists in the micromolar range (O'Leary et al., 2008). In recent years, we and others have proposed that cotinine functions as a positive allosteric modulator of α7 nAChRs (Buccafusco et al., 2009; Zeitlin et al., 2012). Positive allosteric modulators, are compounds that facilitate endogenous neurotransmission without directly stimulating the target receptors (Bertrand and Gopalakrishnan, 2007; Faghih et al., 2007). For example the compound PNU-120596, acts as a positive allosteric modulator of nAChRs both *in vitro* and *in vivo* (see Young et al., 2008). PNU-120596 inhibited the ability of amphetamine to suppress auditory gating in rats, suggesting its potential for use in schizophrenia, characterized by auditory gating deficits. The effects of PNU-120596 *in vitro* were shown to be mediated by α7 nAChRs. As a positive allosteric modulator, PNU-120596 positively modulates nicotinic cholinergic neurotransmission mostly by preventing receptor desensitization. Because, cotinine has been shown to be a memory enhancer (Echeverria et al., 2011), and also inhibit sensory gating disruption it has been speculated that can have similar beneficial effects on memory in AD or schizophrenia (Hajos and Rogers, 2010). However this is highly speculative at this point and needs to be demonstrated.

Another hypothesis proposed to explain the beneficial effects of cotinine on cognition is the theory of desensitization of specific population of the α7nAChRs. This hypothesis speculates that cotinine desensitization of the α7 nAChRs expressed on inhibitory GABAergic neurons of the hippocampus, may result in the activation of excitatory glutamate receptors mediating the synaptic plasticity changes required for memory (Buccafusco et al., 2007, 2009). Functional assays of human α7 nAChR expressed in *Xenopus leavis* oocytes showed that cotinine acted as weak agonists at the human α7 nAChR (1% response at 1 mM) and inhibited the response to ACh with IC_{50} value of 175 μM (Briggs and McKenna, 1998). Although this hypothesis is intriguing, the doses required for inducing the receptor desensitization are higher than those showing pharmacological effects and direct evidence that cotinine may have this effect in the brain is still missing. The idea of a desensitization of the α7nAChR induced by cotinine is contradictory with the fact that in mice, the chronic treatment with cotinine induced the activation of the Akt/GSK3β signaling pathway, which is activated by the α7 nAChR, in both the hippocampus and cortex (Echeverria et al., 2011). In fact, cotinine prevented apomorphine-induced deficits in PPI of acoustic startle in rats (Buccafusco and Terry, 2003), a behavioral task that greatly depends on the activity of the α7 nAChR. The effects of cotinine can be better explained by cotinine functioning as a positive allosteric modulator of the human α7 nAChR.

As a positive modulator, cotinine may improve learning and memory performance and reverse the apomorphine-induced deficits of PPI, in addition to stimulating the protein kinase B (Akt)/GSK3β pathway. Furthermore, stimulation of α7 nAChR signaling may explain the neuroprotective effects of cotinine because Akt can promote neuronal survival via several mechanisms including stimulation of the expression of anti-apoptotic factors such as CREB and Bcl-2 while inactivating pro-apoptotic enzyme such as Ask1 (Kim et al., 2001). Evidence of an allosteric effect of cotinine on the α7 nAChR or other receptors.

CONCLUSION

Altogether the evidence suggests that cotinine is less toxic and has different mechanism(s) of action than nicotine. Cotinine's properties and the preclinical evidence of its nootropic effects in animal models of psychiatric conditions, suggests that cotinine as a pure agent, in absence of nicotine, represents a new therapeutic

agent to reduce anxiety, facilitate the extinction of fear memories, and improve attention and working memory in individuals with psychiatric conditions such as AD.

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Beta amyloid differently modulate nicotinic and muscarinic receptor subtypes which stimulate *in vitro* and *in vivo* the release of glycine in the rat hippocampus

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Using both *in vitro* (hippocampal synaptosomes in superfusion) and *in vivo* (microdialysis) approaches we investigated whether and to what extent β amyloid peptide 1–40 (Aβ 1–40) interferes with the cholinergic modulation of the release of glycine (GLY) in the rat hippocampus. The nicotine-evoked overflow of endogenous GLY in hippocampal synaptosomes in superfusion was significantly inhibited by Aβ 1–40 (10 nM) while increasing the concentration to 100 nM the inhibitory effect did not further increase. Both the Choline (Ch; α7 agonist; 1 mM) and the 5-Iodo-A-85380 dihydrochloride (5IA85380, α4β2 agonist; 10 nM)-evoked GLY overflow were inhibited by Aβ 1–40 at 100 nM but not at 10 nM concentrations. The KCl evoked [^{3}H]GLY and [^{3}H]Acetylcholine (ACh) overflow were strongly inhibited in presence of oxotremorine; however this inhibitory muscarinic effect was not affected by Aβ 1–40. The effects of Aβ 1–40 on the administration of nicotine, veratridine, 5IA85380, and PHA543613 hydrochloride (PHA543613; a selective agonist of α7 subtypes) on hippocampal endogenous GLY release *in vivo* were also studied. Aβ 1–40 significantly reduced (at 10 μM but not at 1 μM) the nicotine-evoked *in vivo* release of GLY. Aβ 1–40 (at 10 μM but not at 1 μM) significantly inhibited the PHA543613 (1 mM)-elicited GLY overflow while was ineffective on the GLY overflow evoked by 5IA85380 (1 mM). Aβ 40–1 (10 μM) did not produce any inhibitory effect on nicotine-evoked GLY overflow both in the *in vitro* and *in vivo* experiments. Our results indicate that (a) the cholinergic modulation of the release of GLY occurs by the activation of both α7 and α4β2 nicotinic ACh receptors (nAChRs) as well as by the activation of inhibitory muscarinic ACh receptors (mAChRs) and (b) Aβ 1–40 can modulate cholinergic evoked GLY release exclusively through the interaction with α7 and the α4β2 nAChR nicotinic receptors but not through mAChR subtypes.

Keywords: β amyloid, glycine release, nicotinic receptors, muscarinic receptors, microdialysis

INTRODUCTION

Nicotinic and muscarinic receptors are widely expressed in the brain and implicated in the pathophysiology of many neurological conditions, including Alzheimer's disease (AD), where typical symptoms include the loss of cognitive function and dementia. The presence of extracellular neuritic plaques composed of β amyloid (Aβ) peptide is a characteristic feature of AD, however, although neurotoxicity is a prominent feature of Aβ, recent data emphasize the existence of synaptic functional roles of this peptide which can modulate the release of several neurotransmitters (see Mura et al., 2012 and references therein). Accordingly, Aβ isoforms and oligomers of increasing molecular dimensions may have different biological actions in a continuum from physiology to pathology, determining loss and gains of function along the course of the disease (Mura et al., 2010a). Indeed, it has been shown that non-neurotoxic Aβ 1–40 concentrations were able to modulate (predominantly, but not exclusively, to inhibit) the release of several neurotransmitters (dopamine, γ aminobutyric acid, aspartate, glutamate) elicited by the stimulation of cholinergic muscarinic and nicotinic receptor [muscarinic ACh receptors (mAChR); nicotinic ACh receptors (nAChR)] subtypes in different brain areas; (Preda et al., 2008; Puzzo et al., 2008; Grilli et al., 2009; Jürgensen and Ferreira, 2010;Mura et al., 2010a,b, 2012; Ondrejcak et al., 2010). The hippocampus, an area which is particularly vulnerable and early target of Alzheimer's disease and in which the cholinergic pathways are critical for modulation of attention and memory (Parri et al., 2011), Aβ regulates the nicotine-evoked release of both excitatory (glutamate and aspartate) and inhibitory (γ aminobutyric acid) aminoacids (Mura et al., 2012).

Increasing evidence demonstrate that glycine (GLY) is an important aminoacid at hippocampal level which may have a dual role acting as an inhibitory neurotransmitter, when interacting with the strychnine-sensitive receptors, and playing a stimulatory role, when co-activating excitatory *N*-Methyl-D-aspartic acid receptors together with glutamate (Johnson and Ascher, 1987; Luccini et al., 2008; Romei et al., 2009, 2011; Zappettini et al., 2011). No

data are available so far on the possible effects of Aβ on the cholinergic receptors which modulate GLY release at the hippocampal level.

In the present study using both *in vitro* (hippocampal synaptosomes in superfusion) and *in vivo* (microdialysis) approaches we investigated whether and to what extent Aβ interferes with the cholinergic modulation of the release of GLY in the rat hippocampus.

The results indicate that (a) the cholinergic modulation of the release of GLY occurs by the activation of both α7 and α4β2 (nAChRs) as previously reported (Zappettini et al., 2011) as well as by the activation of inhibitory mAChRs; (b) the nicotinic modulation of GLY release by α4β2, and α7 nAChRs is inhibited *in vitro* in presence of a nanomolar concentration of Aβ 1–40 which is on the contrary ineffective on the inhibitory mAChR receptor subtypes.

MATERIALS AND METHODS

ANIMALS

Adult male Wistar rats (200–250 g, Harlan, Udine) were used for both *in vivo* experiments and as brain tissue source for *in vitro* experiments. Animals were housed at constant temperature (22 ± 1°C) and relative humidity (50%) under a regular light–dark schedule (light 7 a.m. to 7 p.m.). The *in vitro* experimental procedures were approved by the Ethical Committee of the Pharmacology and Toxicology Section, Department of Experimental Medicine, in accordance with the European legislation (European Communities Council Directive of 24 November 1986, 86/609/EEC) and were approved by Italian legislation on animal experimentation (Decreto Ministeriale number 124/2003-A). The *in vivo* protocol was approved by Ethical Committee of Pavia's University (registered as 2/2008) according to international regulations for the care and treatment of laboratory animals, to the Italian Act (DL n 116, GU, suppl 40, 18 February, 1992) and to EEC Council Directive (86/609, OJ L 358, 1, 12 December, 1987). All efforts were made to minimize animal suffering and to use the minimal number of animals necessary to produce reliable results.

IN VITRO EXPERIMENTS

Experiments of release

Rats were killed by decapitation and the hippocampus rapidly removed at 0–4°C. Purified synaptosomes were prepared on Percoll® gradients (Sigma-Aldrich, St Louis, MO, USA) essentially according to (Nakamura et al., 1993), with only minor modifications. Briefly, the tissue was homogenized in six volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris–HCl, using a glass-teflon tissue grinder (clearance 0.25 mm, 12 up–down strokes in about 1 min). The homogenate was centrifuged (5 min, 1000 × *g* at 4°C) to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll® gradient (2, 6, 10, and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min at 4°C. The layer between 10 and 20% Percoll® (synaptosomal fraction) was collected, washed by centrifugation, and resuspended in physiological HEPES-buffered medium having the following composition (mM): NaCl 128, KCl 2.4, $CaCl_2$ 3.2, KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 25, pH 7.5, glucose 10, pH 7.2–7.4 (Lu et al., 1998). Synaptosomal protein content following purification was 10–15% of that in the supernatant stratified on the Percoll® gradient.

The synaptosomal suspension was layered on microporous filters at the bottom of a set of parallel superfusion chambers maintained at 37°C (Raiteri and Raiteri, 2000; Superfusion System, Ugo Basile, Comerio, Varese, Italy). Synaptosomes were superfused at 1 ml/min with standard physiological medium as previously described. The system was first equilibrated during 36.5 min of superfusion; subsequently, four consecutive 90 s fractions of superfusate were collected and the endogenous GLY content was measured by high performance liquid chromatography as below described. Synaptosomes were exposed to agonists for 90 s starting from the second fraction collected ($t = 38$ min), with antagonists being added 8 min before agonists. The evoked overflow was calculated by subtracting the corresponding basal release from each fraction and was expressed as pmol/mg of synaptosomal proteins. We previously demonstrated that in our superfusion system the indirect drug effects exerted by other mediators in the monolayer of synaptosomes in superfusion are absolutely minimized (Raiteri and Raiteri, 2000).

When studying the release of [^{3}H]GLY or [^{3}H]Acetylcholine (ACh) hippocampal synaptosomes were incubated for 20 min at 37°C with [^{3}H]GLY (final concentration 0.1 μM) in the presence of the selective GLY transporter 1 transporter blocker *N*-[(3R)-3-([1,1′-biphenyl]-4-yloxy)-3-(4-fluorophenyl)propyl]-*N*-methylglycine hydrochloride (final concentration 0.3 μM) or with [^{3}H]Choline (Ch, final concentration 0.08 μM). The K^+-induced overflow from synaptosomes was estimated by subtracting the neurotransmitter content into the first and the third fractions collected (basal release, b1 and b3) from that in the 6-min fraction collected during and after the depolarization pulse (evoked release, b2). The amount of radioactivity released into each superfusate fraction was expressed as a percentage of the total synaptosomal tritium content at the start of the fraction collected (fractional efflux).

ENDOGENOUS GLY DETERMINATION

Endogenous GLY was measured by high performance liquid chromatography analysis following precolumn derivatization with *o*-phthalaldehyde and resolution through a C18-reverse phase chromatographic column (10 mm × 4.6 mm, 3 μm; Chrompack, Middleburg, The Netherlands) coupled with fluorometric detection (excitation wavelength 350 nm; emission wavelength 450 nm). Homoserine was used as internal standard. Buffers and gradient program were prepared and executed as follows: solvent A, 0.1 M sodium acetate (pH 5.8)/methanol, 80:20; solvent B, 0.1 M sodium acetate (pH 5.8)/methanol, 20:80; solvent C, sodium acetate (pH 6.0)/methanol, 80:20; gradient program, 100% C for 4 min from the initiation of the program; 90% A and 10% B in 1 min; 42% A and 58% B in 14 min; 100% B in 1 min; isocratic flow 2 min; 100% C in 3 min; flow rate 0.9 ml/min.

IN VIVO EXPERIMENTS

Microdialysis probe implantation

Rats were anesthetized with Equithesin 3 ml/kg (pentobarbital 9.7 g, chloral hydrate 42.5 g, $MgSO_4$ 21.3 g for 1 l, 10% ethanol, 40% propylene glycol v/v) administered intraperitoneally and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA). The skin was shaved, disinfected, and cut

with a sterile scalpel to expose the skull. A hole was drilled to allow the implantation of the probe into the brain parenchyma. The probe was implanted in the hippocampus (CA1/CA2 regions; AP −5.8 mm, ML ± 5.0 mm from bregma, and DV −8.0 mm from dura) according to the Paxinos and Watson (1986) atlas, and secured to the skull with one stainless steel screw and dental cement. All *in vivo* experiments were performed using microdialysis probes, made in our laboratory according to the original method described by Di Chiara (1990; Emophan Bellco Artificial OR-internal diameter 200 μm, cutoff 40 kDa; Bellco, Mirandola, Modena, Italy), with a nominal active length of 5 mm. Finally, the skin was sutured, and the rats were allowed to recover from anesthesia for at least 24 h before the neurotransmitter release study.

Microdialysis samples collection

Microdialysis experiments were performed on conscious freely moving rats. On the day of the experiments (24 h after the surgical procedure), the probe was perfused with artificial CSF containing 145 mM NaCl, 3.0 mM KCl, 1.26 mM $CaCl_2$, 1.0 mM $MgCl_2$, 1.4 mM Na_2HPO_4, buffered at pH 7.2–7.4, and filtered through a Millipore 0.2 μm pore membrane. In all experiments, the microdialysis membrane was allowed to stabilize for 1 h at the flow rate of 4 μl/min, without collecting samples. At the end of the stabilization period, three samples were collected to evaluate baseline release of GLY and then the specific treatment started. All treatments were administered by manually switching syringes and tubing connections to allow drugs diluted in artificial CSF to flow through the probes. Tubing switches were performed taking care to maintain constant flow rates and collection volumes. Both basal and treatment samples were collected every 20 min in 100 μl Eppendorf tubes. The flow rate of 4 μl/min was maintained using a 1000-μl syringe (Hamilton) and a microinjection pump (CMA/100, CMA/Microdialysis AB). *In vitro* recovery of the probe for GLY was about 20%. Each rat was used for only one microdialysis session. At the end of each experiment animals were sacrificed by guillotine, rat brains were removed and the position of the microdialysis probe was verified by histological procedures, slicing the tissues by a cryostat microtome (LEICA CM 1510). Only data from rats in which probe tracks were exactly located in the target area were used for statistical analysis.

Immunohistochemical analysis

Immunohistochemical analysis was performed to verify the presence of Aβ in the perfused tissue and to confirm (according to HOECHST 33342 staining) the absence of neurotoxic-induced apoptotic phenomenon. Brain tissue samples were frozen and stored at −80°C. For immunodetection of infused Aβ peptide, 10 μm coronal sections (obtained on a cryostat Leica CM 1510) were incubated with a primary monoclonal antibody recognizing Aβ protein (clone 4G8; Chemicon International). Sections were then incubated with a mouse anti-IgG antibody RPE conjugated (Dako). After the fluorescent labeling procedures, sections were finally counterstained for DNA with HOECHST 33342 and mounted in a drop of Mowiol (Calbiochem, Inalco SpA, Milan, Italy). Fluorescent micrographs were acquired with a Leica TCS SP5 II confocal microscope. After acquisition of fluorescent micrographs, the slides were demounted and then the same sections were slightly counterstained with Mayer hematoxylin, dehydrated, and mounted in DPX for microanatomical analysis. The images were acquired with a BX51 Olympus microscope.

STATISTICAL ANALYSIS

In vitro experiments

Multiple comparisons were performed with one-way ANOVA followed by an appropriate *post hoc* test (Dunnett and Bonferroni). Data were considered significant for $p < 0.05$, at least.

In vivo experiments

Values were expressed either as amount of GLY measured in the dialyzate (pmol/80 μl) or as area under the curve (AUC), evaluating the cumulative release over time. AUC was used as a measure of treatment exposure and was calculated, for each animal, using GraphPad Prism (version 4.03 GraphPad Software, San Diego, CA, USA), defining as baseline of the area the basal value (average concentration of three consecutive samples immediately preceding the drug dose).

D'Agostino–Pearson Omnibus Test (GraphPad Prism, version 4.03, GraphPad Software, San Diego, CA, USA) and Grubb's Test (GraphPad QuickCalcs, online calculator for scientists at http://www.graphpad.com/quickcalcs/, GraphPad Software, San Diego, CA, USA) were used as preliminary tests in order to evaluate whether data were sampled from a Gaussian distribution and to detect outliers respectively. All outliers were excluded from the analysis. Data were then analyzed by analysis of variance (one- or two-way ANOVA) followed, when significant, by an appropriate *post hoc* comparison test. Data were considered significant for $p < 0.05$. The reported data are expressed as means ± SEM. The number of animals used for each experiment is reported in the legend to figures.

PREPARATION OF BETA AMYLOID SOLUTIONS

In the case of both *in vivo* and *in vitro* experiments, synthetic human Aβ 1–40 (Sigma-Aldrich, Milan, Italy) was dissolved in aCSF at a concentration of 100 mM (stock solution). Then, this solution was filtered through a Millipore 0.2 μm pore membrane and stocked in small aliquots. Working solutions were freshly prepared by diluting an aliquot of Aβ 1–40 stock solution at the final concentrations (10 mM, 1 mM, or 100 nM Aβ 1–40 for *in vivo* experiments, 100 nM, 10 nM, 1 nM, or 100 pM for *in vitro* analysis).

CHEMICALS

Beta amyloid (1–40; 40–1), percoll®, choline, himbacine, dimethyl sulfoxide, veratridine, nicotine hydrogen tartrate salt (Sigma-Aldrich, St Louis, MO, USA); NFPS (ALX 5407), 5IA85380, PHA543613, AQRA741 (Tocris Bioscience, Bristol, UK); all salts used for the preparation of aCSF (NaCl, KCl, $CaCl_2$, $MgCl_2$, Na_2HPO_4) and for Equithesin ($MgSO_4$) were purchased at Merck KGaA, Darmstadt, Germany; chloral hydrate, ethanol 96%, and propylene glycol were used for the preparation of Equithesin and were obtained at VWR BDH Prolabo, Belgium;[^{3}H]Choline (specific activity: 60–90 Ci/mmol) and [^{3}H]Glycine (specific activity: 15 Ci/mmol) were purchased from Perkin Elmer SpA.

RESULTS

It is known that Aβ peptides structure and aggregation properties depend on several factors. It is therefore important when using Aβ peptides to specify both the concentration of the soluble Aβ but also the methods used to prepare Aβ solution and to verify the presence of Aβ aggregates (see Parri et al., 2011). This facilitates the interpretation of the results when reporting on Aβ effects and, which is even more important, would facilitate the comparison of data obtained in different laboratories. Hence, in our previous paper we characterized the Aβ peptide conformation we administered *in vivo* by Western Blot procedure (Mura et al., 2012) showing that we administered, at least predominantly, Aβ monomers. We cannot completely exclude by the adopted methods that small amounts of Aβ oligomers are present and may participate to produce the observed effects. In regard to the *in vitro* Aβ preparations, since we did not observe aggregation at the concentrations and the timing (up to 40 min) analyzed *in vivo*, we also do not expect to observe aggregation at the lower concentrations and shorter times used *in vitro* in light of the fact that aggregation is a concentration and time-dependent process.

Figure 1A shows that the nicotine-evoked overflow of endogenous GLY was significantly inhibited by Aβ 1–40 already at a concentration of 10 nM while increasing the concentration to 100 nM the inhibitory effect did not further increase. The 1-nM Aβ 1–40 concentration was ineffective. The reverse peptide Aβ 40–1 was ineffective even at high (100 nM) concentration. Aβ 1–40 (100 nM) did not affect the veratridine (10 nM)-evoked GLY overflow.

Since it has been shown that two different nAChR subtypes, α7 and α4β2, modulate GLY release (Zappettini et al., 2011), the possibility that Aβ may differentially inhibit the nicotinic control of GLY release has been investigated. In order to verify this point we have studied the effects of two different agonists, Ch and 5-IA85380 hydrochloride (5IA85380), known to act selectively on the α7 and α4β2 nAChR subtypes respectively (Mukhin et al., 2000; Uteshev et al., 2003; Dickinson et al., 2007; Zappettini et al., 2010). Ch (1 mM) and the 5IA85380 (10 nM)-evoked a similar overflow of GLY confirming the involvement of the two receptor subtypes. Both the Ch (1 mM) and the 5IA85380 (10 nM)-evoked GLY overflow were significantly inhibited by Aβ 1–40 at 100 nM but not at 10–1 nM concentrations compared to controls (**Figure 1B**). Aβ 1–40 (100 nM) did not modify the basal release of endogenous GLY (data not shown).

It has been demonstrated that also different mAChR subtypes are involved in the modulation of both ACh and GLY release from brain hippocampal synaptosomes (Raiteri et al., 1984; Russo et al., 1993). We investigated whether Aβ was able to affect the muscarinic control of the release of these two transmitters as it was able to disrupt the nicotinic control of GLY release. **Figure 2A** shows that the KCl evoked [^{3}H]GLY overflow was strongly inhibited in presence of oxotremorine. This inhibitory effect was totally antagonized by himbacine but was not affected by Aβ 1–40 (100 nM). In presence of oxotremorine also the KCl evoked release of [^{3}H]ACh from hippocampal nerve endings was significantly inhibited (**Figure 2B**). This result was unexpected but demonstrates quite interestingly that the cholinergic muscarinic modulation of GLY release could be different according to the different brain areas or the different species studied since previous findings using human cortical nerve endings have shown a potentiating effect of oxotremorine on GLY release (Russo et al., 1993). Interestingly also the inhibitory effect of oxotremorine on [^{3}H]ACh release was antagonized, as expected, by the specific M2 mAChR antagonist AQRA741 but was not affected by Aβ 1–40 (100 nM; **Figure 2B**).

Based on the *in vitro* data we then analyzed the effects of Aβ 1–40 on the administration of nicotine, veratridine, 5IA85380, and PHA543613 hydrochloride (PHA543613, a selective agonist of α7 subtypes) on hippocampal GLY release *in vivo*. In order to test whether the administration of Aβ 1–40 through the dialysis probe allowed the delivery of the peptide to the tissue we performed an immunohistochemical analysis. **Figure 3** shows the presence of the peptide for the two concentrations tested *in vivo* (1 and 10 μM) within the hippocampus. Despite the fact that we do not know the exact amounts of Aβ reaching the tissue, there was a visible positive correlation between the concentration administered and the signal of Aβ immunoreactivity in the tissue. Moreover, immunohistochemical analysis shows that no evident signs of apoptosis were observed within the area of amyloid diffusion as shown by Hoechst staining.

The choice of the concentration of the nicotinic cholinergic agonists to be delivered *in vivo* was derived from previous data demonstrating that the administration by microdialysis of 50 mM nicotine was able to significantly increase the levels of GLY in hippocampal extracellular compartment (Toth, 1996; Fedele et al., 1998; Zappettini et al., 2011). As previously shown in our experimental conditions 40 min-long administration of 50 mM nicotine was able to greatly enhance GLY release from basal values. **Figure 4A** show that Aβ 1–40 significantly reduced (at 10 μM but not at 1 μM) the nicotine-evoked *in vivo* release of GLY. Aβ 40–1 did not produce any inhibitory effect used at 10 μM concentration. The GLY overflow stimulated by veratridine was unaltered in presence of Aβ 1–40 (**Figure 4B**). Then we compared the effects Aβ 1–40 after exposure to the selective nAChRs agonists, 5IA85380, and PHA543613. As shown in **Figure 4C**, Aβ 1–40 (at 10 μM but not at 1 μM) significantly inhibited the PHA543613 (1 mM)-elicited GLY overflow while was ineffective on the GLY overflow evoked by 5IA85380 (1 mM).

DISCUSSION

It is expected that cognitive deficits and memory impairments in AD patients could be related, at least in part, to Aβ mediated decrease of cholinergic function (Wang et al., 2009a,b, 2010; Parri et al., 2011). However it is still unclear whether these impairments are a consequence of a loss of cholinergic neurons and a decrease of nAChRs or of a direct molecular interaction of Aβ with nAChRs leading to a dysregulation of receptor function or of both mechanisms. To shed some light on these mechanisms we investigated in the present study as well as in previous researches whether Aβ, at concentrations not producing acutely neurotoxicity, is able to disrupt the cholinergic control of neurotransmitter release. To do this we took advantage of the fact that both α4β2, and α7 nAChRs receptors are known to be expressed in rat hippocampal interneurons (McQuiston and Madison, 1999; Sudweeks and Yakel, 2000; Yakel and Shao, 2004), and it is well-known that they have a positive

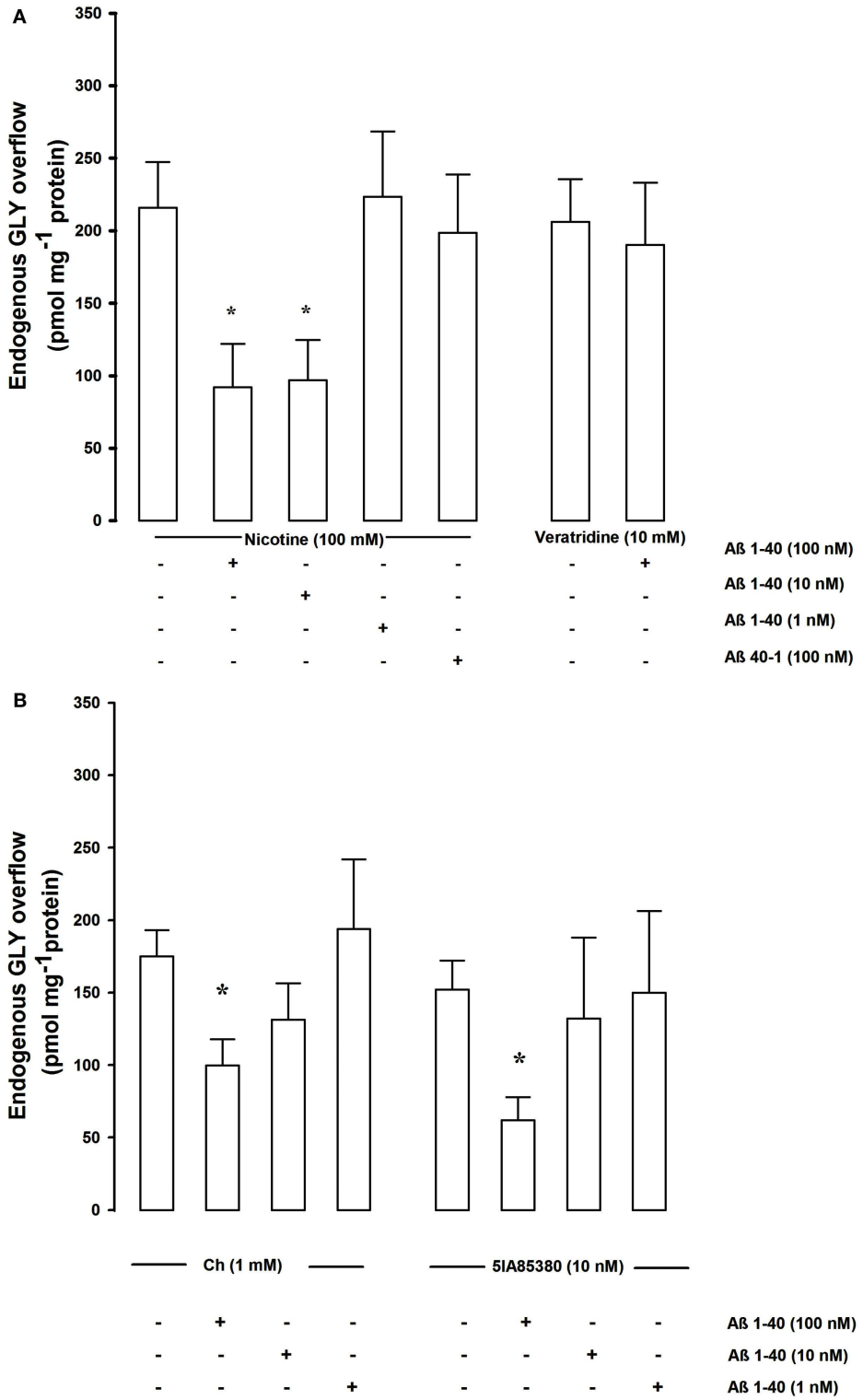

FIGURE 1 | (A) Shows the concentration dependence effects of Aβ on nicotine- and veratridine-evoked endogenous GLY overflows from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments for each concentration run in triplicate. $*p < 0.05$ versus nicotine-evoked GLY overflow (one-way ANOVA followed by Bonferroni *post hoc* Test). **(B)** Shows the concentration dependence effects of Aβ on Ch and 5IA85380 evoked endogenous GLY overflows from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments for each concentration run in triplicate. $*p < 0.05$ versus Ch-evoked GLY overflow (one-way ANOVA followed by Dunnett's Multiple Comparison Test).

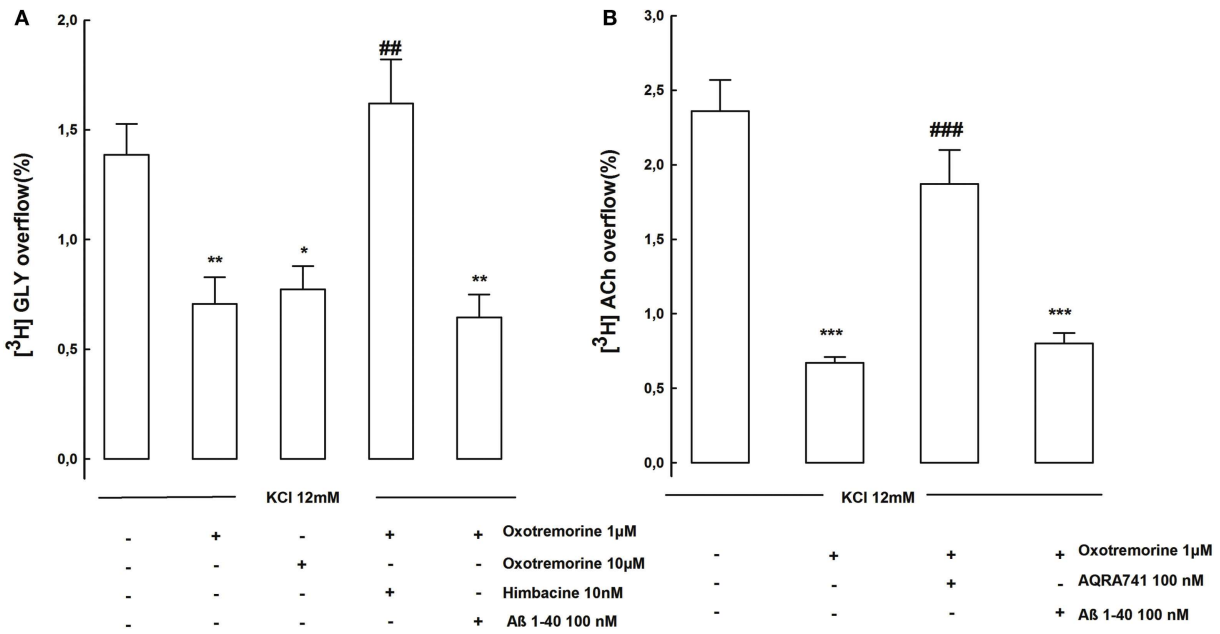

FIGURE 2 | (A) Shows the effects of Aβ on muscarinic receptors controlling KCl evoked [^{3}H]GLY overflow from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments run in triplicate. *$p < 0.05$; **$p < 0.01$ versus KCl evoked [^{3}H]GLY overflow; $^{\#\#}p < 0.01$ versus KCl plus oxotremorine (1 μM)-evoked [^{3}H]GLY overflow. **(B)** Shows the effects of Aβ on muscarinic receptors controlling KCl evoked [^{3}H]ACh overflow from rat hippocampal synaptosomes. Data are mean ± SEM of three to six experiments run in triplicate. ***$p < 0.001$ versus KCl evoked [^{3}H]ACh overflow; $^{\#\#}p < 0.001$ versus KCl plus oxotremorine (1 μM) -evoked [^{3}H]ACh overflow (one-way ANOVA followed by Bonferroni *post hoc* Test).

role in regulating cognitive function (Picciotto et al., 1995; Levin and Simon, 1998). Being aware that several neurotransmitters that play important roles in cognitive functions could be affected either directly or indirectly by Aβ, we focused our attention on GLY. Indeed increasing evidence demonstrate that GLY is an important aminoacid at hippocampal level which has a dual role (a) acting as an inhibitory neurotransmitter when interacting with the strychnine-sensitive receptors and (b) playing a fundamental stimulatory role when co-activating excitatory *N*-Methyl-D-aspartic acid receptors together with glutamate (Johnson and Ascher, 1987; Hirai et al., 1996; Luccini et al., 2008; Kubota et al., 2010; Zappettini et al., 2011). The changes in the GLY release may therefore directly interfere with glutamate neurotransmission, which plays an important role in the processes of learning and memory in this brain area.

We here report that the cholinergic modulation of GLY release at the presynaptic level on hippocampal nerve endings was modulated not only by stimulatory α4β2 and α7 nAChRs as previously reported (Zappettini et al., 2011) but also by an inhibitory, mAChR subtype. We do not know whether both nicotinic and muscarinic receptors are present on all nerve endings or they are peculiar of a specific neuronal population and/or their physiological importance in the intact tissue. It is quite interesting however that the two modulatory mechanisms display a different sensitivity to Aβ. The stimulatory effects of both α4β2 and α7 nAChRs were partially blocked by nanomolar concentration of Aβ 1–40 which was on the contrary inactive on the mAChRs inhibiting GLY release. This observation allows to speculate that an excess of Aβ (the range of active concentrations was between 10 and 100 nM *in vitro* and 10 μM *in vivo*) as it may happen because of the disease may dysregulate the cholinergic modulation of hippocampal activity leading to a disproportionate inhibition since it leaves unaffected the muscarinic inhibitory control and impairs the nicotinic stimulatory one. All this occurs in absence of Aβ acutely induced neuronal damage.

The decreased release of GLY may have several functional consequences. Some of them may be relevant to the development of AD pathology. As an example, a decrease of GLY release may reduce the tonic inhibition exerted through the activation of GLY receptors (Mori et al., 2002; Petrini et al., 2004; Farrant and Nusser, 2005) normally providing neuroprotection under pathological conditions, when extracellular GLY levels are elevated (Baker et al., 1991; Saransaari et al., 1997; Zhao et al., 2005; Saransaari and Oja, 2008). A second relevant event caused by a reduced glycine release may consist in a decrease of *N*-Methyl-D-aspartic acid receptors co-activation. At this regard it is important to recall that intracerebroventricular injection of Aβ 1–40 significantly suppress high frequency stimulation-induced LTP (Chen et al., 2006; Wu et al., 2008).

The exact nature of the Aβ interaction with nAChR subtypes is so far not well understood.

Interestingly in our study both the α7 and the α4β2 nAChR subtypes which almost equally contribute to the stimulation of GLY release, were functionally inhibited *in vitro* apparently in a similar extent by Aβ concentrations in the nanomolar range (**Figure 2B**). However we do not know whether Aβ might have

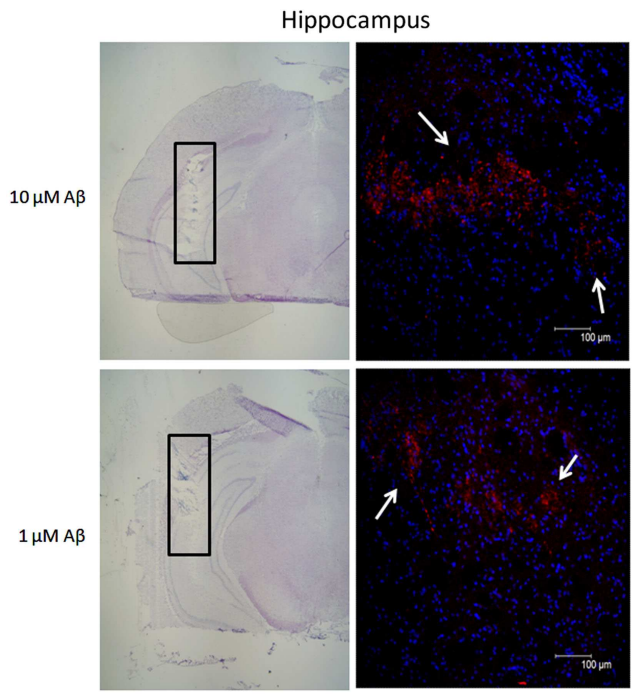

FIGURE 3 | Immunohistochemical analysis showing the presence of beta-amyloid (Aβ) in hippocampal tissue after the perfusion of the peptide at two different concentrations. Coronal section indicating the location of the microdialysis probe (hippocampus, black rectangle) counterstained with Mayer Hematoxylin and relative fluorescence micrographs of the area within the black rectangle showing the presence of human Aβ protein. Aβ immunoreactivity (red-PE staining, white arrows) immediately after perfusion of 10 and 1 µM Aβ 1–40. Nuclear DNA was counterstained with Hoechst 33,342 (blue staining). Scale bars for Mayer hematoxylin sections: 200 µM. Scale bars for fluorescent micrographs: 100 µM.

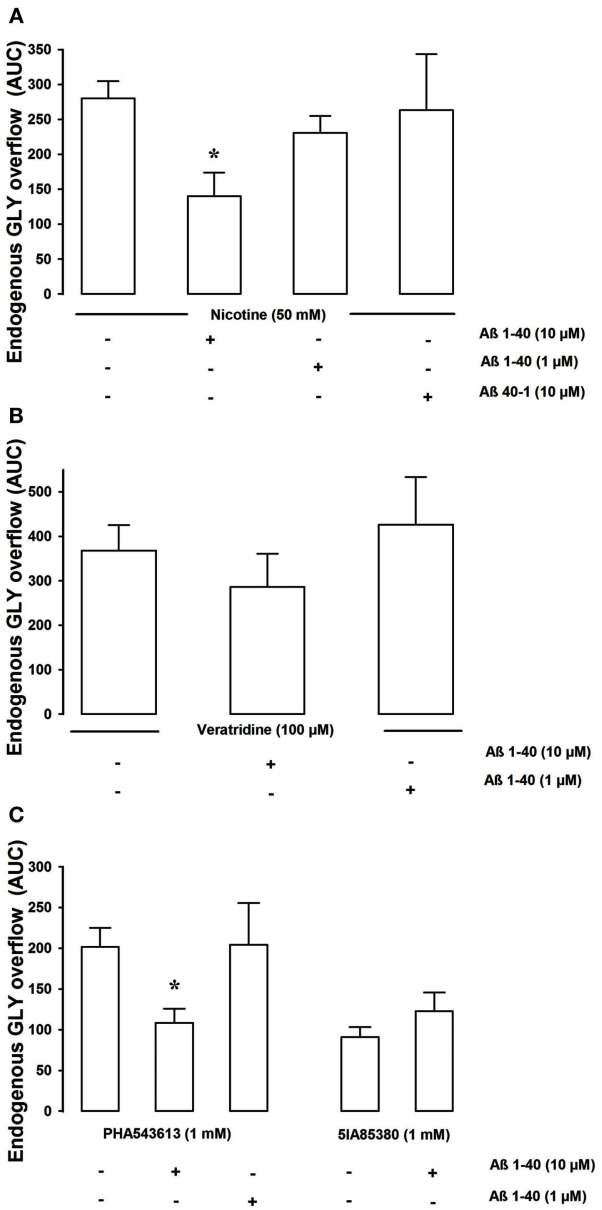

FIGURE 4 | The figure shows the *in vivo* effect of Aβ 1–40 on the nicotine-, veratrine-, PHA543613-, and 5IA85380-induced overflow of GLY, in rat hippocampus [(A–C) respectively]. Aβ 1–40 (10 µM but not 1 µM) significantly inhibited the 50-mM nicotine-induced overflow of GLY $^*p < 0.01$ versus nicotine (one-way ANOVA followed by Dunnett's Multiple Comparison Test). Aβ 40–1 10 µM was ineffective **(A)**. Aβ 1–40 (10 µM) which significantly inhibited the 1-mM PHA543613-induced overflow of GLY was unable to inhibit both the 100-µM veratridine- and the 5IA85380-induced overflow of GLY **(B,C)**. Data are expressed as mean ± SEM of 4–15 individual rats for each experimental group. $^*p < 0.01$ versus PHA543613 (one-way ANOVA followed by Dunnett's Multiple Comparison Test.

a similar mechanism of action on the two different nAChR subtypes. The inhibitory effect on both the α7 and the α4β2 nAChR was incomplete with a maximal inhibition of about 30–40%. A recently described allosteric binding pocket located within the trans membrane domain of the α7 and of non-α7 nAChRs might provide a potential structure-function mechanism to explain the inhibitory effects of Aβ (Young et al., 2008; Gill et al., 2011, 2012).

Indeed Aβ at concentration in the upper nanomolar range (10 nM) produced full effect while did not produced any effect at 1 nM concentration. Ten nanomolars Aβ beyond the estimated normal concentrations in human CSF but may be caused by the altered precursor protein processing as it occurs along with the disease (Reaume et al., 1996) or be related to defects in the removal of the peptide from the extracellular space as it may occur in the disease in association with the ApoE ε4 genotype (Cramer et al., 2012).

Of course talking about cholinergic modulation of GLY release we have also to consider the possibility that Aβ might for instance interfere directly with the mechanisms which modulate the release of ACh. However our data show that Aβ was ineffective also on the inhibitory muscarinic autoreceptors which inhibit ACh release (**Figure 2**). We can therefore foresee that in an integrated system, where cellular networks and their functional relationships are completely preserved and several direct and indirect processes are

simultaneously taking place in neurons, the effect of Aβ on the cholinergic modulation of GLY function may mostly depend on the interaction of Aβ with the nAChRs.

Our findings *in vivo* largely support this view even if Aβ was unable to inhibit the release of GLY elicited by the specific α4β2 nAChRs agonist while it exerted an inhibition (over 40%) of both nicotine and PHA543613 stimulated GLY release. On the other hand *in vivo* important differences were noticed at the baseline in the ability of the two specific nAChRs agonists to elicit GLY release as compare to *in vitro*. Indeed *in vitro* both α7 and α4β2 nAChRs agonists were able to stimulate to the same extent GLY release; while *in vivo* the release elicited by 5IA85380 was less than a half that obtained in response to PHA543613. *In vivo* to many variables may take place, and in spite of the above described discrepancy, there is a general good agreement of the effects *in vivo* and *in vitro* of Aβ on the release of various neurotransmitters (present work and references) including the observation that so far Aβ has never been able to modify the depolarization (veratridine or potassium)-elicited release of neurotransmitters, but only the one modulated by presynaptic receptors.

In conclusion our findings show that Aβ can modulate cholinergic evoked GLY release exclusively through the interaction with α7 and the α4β2 nAChR nicotinic receptors, acting probably through different mechanisms of action, but not through mAChRs. The present study may therefore provide further insight into the mechanism by which AB impairs synaptic plasticity and cholinergic function in AD brain.

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Potential contribution of aromatase inhibition to the effects of nicotine and related compounds on the brain

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Cigarette smoking continues to be a major public health problem, and while smoking rates in men have shown some decrease over the last few decades, smoking rates among girls and young women are increasing. Practically all of the important aspects of cigarette smoking and many effects of nicotine are sexually dimorphic (reviewed by Pogun and Yararbas, 2009). Women become addicted more easily than men, while finding it harder to quit. Nicotine replacement appears to be less effective in women. This may be linked to the observation that women are more sensitive than men to non-nicotine cues or ingredients in cigarettes. The reasons for these sex differences are mostly unknown. Several lines of evidence suggest that many of the reported sex differences related to cigarette smoking may stem from the inhibitory effects of nicotine and other tobacco alkaloids on estrogen synthesis via the enzyme aromatase (cyp19a gene product). Aromatase is the last enzyme in estrogen biosynthesis, catalyzing the conversion of androgens to estrogens. This review provides a summary of experimental evidence supporting brain aromatase as a potential mediator and/or modulator of nicotine actions in the brain, contributing to sex differences in smoking behavior. Additional research on the interaction between tobacco smoke, nicotine, and aromatase may help devise new, sex specific methods for prevention and treatment of smoking addiction.

Keywords: smoking, sex, CYP19, extragonadal estrogen, amygdala, PET imaging, vorozole

INTRODUCTION

Cigarette smoke and nicotine produce diverse behavioral and physiological effects in the developing as well as the adult human brain (Benowitz, 2008), including changes in cognition, anxiety, and aggression. These effects are traditionally explained by an interaction with nicotinic acetylcholine receptors (nAChR). However, some well established peripheral effects of smoking in women are not easily explained by this mechanism. Specifically, female cigarette smokers reach menopause at an earlier age and have lower plasma estrogen levels than non-smoking females (Daniell, 1972; MacMahon et al., 1982; Nusbaum et al., 2000). Female smokers are also at increased risk of osteoporosis, which is a well known correlate of decreased peripheral estrogen levels (Daniell, 1972; Pant and Shapiro, 2008; Korkor et al., 2009).

AROMATASE

Estrogen biosynthesis depends on the enzyme aromatase (Cyp19a gene product, **Figure 1**), which irreversibly converts androgens such as androstenedione and testosterone synthesized in both the ovary and testes, to the estrogens estrone and estradiol, respectively. The mechanism is depicted in **Figure 2**. In humans, the gene is located on chromosome 15q21.1 and is composed of 10 exons. Among them, exons II–X encode the aromatase protein and the 3′-untranslated region of the mRNA, whereas the tissue-specific first exon is alternatively spliced giving rise to a differential 5′-untranslated region of the mRNA in different tissues. Correspondingly, tissue-specific promoters are used for tissue-specific regulation of the *CYP19* gene expression (e.g., Kamat et al., 2002).

Aromatase has a molecular weight of 55 kDa, a K_m for testosterone ranging from ∼60 to ∼240 nmol and a K_m for androstenedione ranging from 0.1 to 30 μM, depending on the source of the enzyme and the lab performing the assay (e.g., Nakajin et al., 1986; Guet et al., 1999; Cooke, 2002; Hong et al., 2007).

NICOTINE AND RELATED ALKALOIDS INHIBIT AROMATASE *IN VITRO*

To explore the possible link between cigarette smoking and decreased endogenous estrogens, Barbieri et al. (1986) examined the effects of constituents of tobacco on estrogen production in human choriocarcinoma cells and term placental microsomes. In choriocarcinoma cell cultures, nicotine, cotinine (a major metabolite of nicotine), and anabasine (a minor tobacco constituent) all inhibited androstenedione conversion to estrogen in a dose-dependent fashion at concentrations in the low micromolar range. Removal of nicotine, cotinine, and anabasine from the culture medium resulted in the complete reversal of the inhibition of aromatase. Furthermore, a supraphysiologic concentration of androstenedione (73 μM) in the culture medium blocked the inhibition of aromatase caused by nicotine, cotinine, and anabasine. In preparations of term placental microsomes, nicotine, cotinine, and anabasine also inhibited the conversion of testosterone to estrogen. Kinetic analysis demonstrated the inhibition to be competitive with respect to the substrate. These findings suggest that tobacco alkaloids exert a direct, competitive, and reversible inhibitory effect on aromatase activity at micromolar concentrations (Barbieri et al., 1986). Importantly, subsequent studies by another research

group discovered additional tobacco constituents and nicotine derivatives with a significantly higher (sub-micromolar concentrations) activity in peripheral tissues including human placenta, similar to that of clinically useful aromatase inhibitors (Osawa et al., 1990; Bullion et al., 1991; Kadohama et al., 1993). To date there have been no published studies of the concentrations of nicotine or its analogs required for *in vitro* inhibition of aromatase in brain cells including astrocytes, microglia, and neurons.

The objective of this review is to summarize recent findings documenting the distribution of aromatase in the brain and its inhibition by nicotine *in vivo*; and to examine the implications these findings may have on our understanding of developmental and acute effects of nicotine on brain physiology and behavior. It is important to note that aromatase inhibition is expected to be mostly similar but not identical to estrogen receptor blockade, since inhibition of aromatase not only lowers the absolute levels of estrogens but also may increase testosterone and thus further reduce the estrogen/testosterone ratio in males and females. Due to sex differences in regional morphology, levels of estrogen, testosterone, and their receptors in the brain (e.g., Cahill, 2005), manipulation of aromatase activity is likely to have sexually dimorphic functional outcomes. Therefore, this review is focused on findings in smoking and in aromatase and aromatase inhibitors rather than the vast body of literature related to effects of estrogen or testosterone on the brain, recently reviewed by McCall and Singer (2012) and by McEwen et al. (2012).

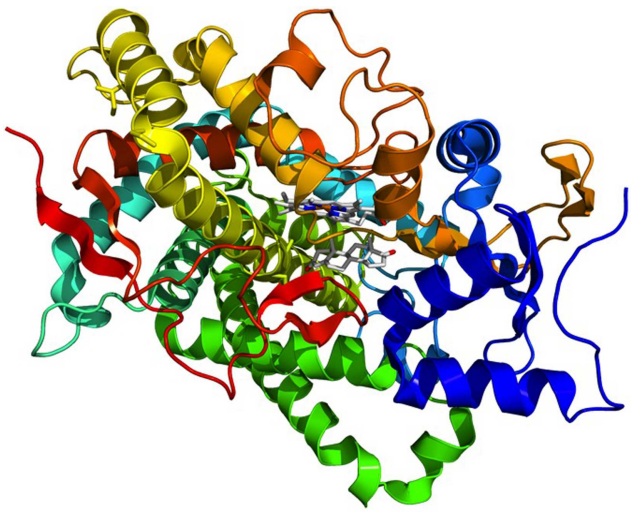

FIGURE 1 | Structure of aromatse. Crystallographic structure of the human aromatase cytochrome P450 (rainbow colored cartoon, N-terminus = blue, C-terminus = red) in complex with the cofactor protoporphyrin IX (top) and the substrate androstenedione (bottom) depicted as stick diagrams (carbon = white, oxygen = red, nitrogen = blue, iron = orange; from Wikipedia).

AROMATASE IS EXPRESSED IN THE ADULT BRAIN AND INHIBITED BY NICOTINE *IN VIVO*

BRAIN EXPRESSION OF AROMATASE

Aromatase expression is found in many brain regions in birds, rodents, non-human primates, and humans (Roselli et al., 1998; Roselli and Resko, 2001; Biegon et al., 2010b; recently reviewed in Biegon et al., 2010b, 2012; Azcoitia et al., 2011). In rodents, the highest concentration of aromatase were found in amygdala and the bed nucleus of the stria terminalis (e.g., Takahashi et al., 2006) with low though significant levels in other regions (e.g., Sierra et al., 2003).

Non-invasive assessment of aromatase availability throughout the primate brain has become possible following radiosynthesis, initial primate studies (Lidstrom et al., 1998; Kim et al., 2009; Biegon et al., 2010a), and validation in humans (Biegon et al., 2010b) of a radiolabeled aromatase inhibitor suitable for positron emission tomography (PET). PET studies in both rhesus monkeys and baboons revealed that the highest levels of aromatase were found in the amygdala and preoptic area while the thalamus and medulla contained low levels (Lidstrom et al., 1998; Roselli and Resko, 2001; Takahashi et al., 2006; Kim et al., 2009).

The most comprehensive study of aromatase distribution in the human brain to date, performed using PET and [^{11}C]vorozole (Biegon et al., 2010b), revealed a highly specific and heterogeneous pattern which appears to be unique to humans. The highest levels were seen in the thalamus, though thalamic distribution was not uniform: within the thalamus, the highest levels were found in the dorsomedial and pulvinar nuclei with lower

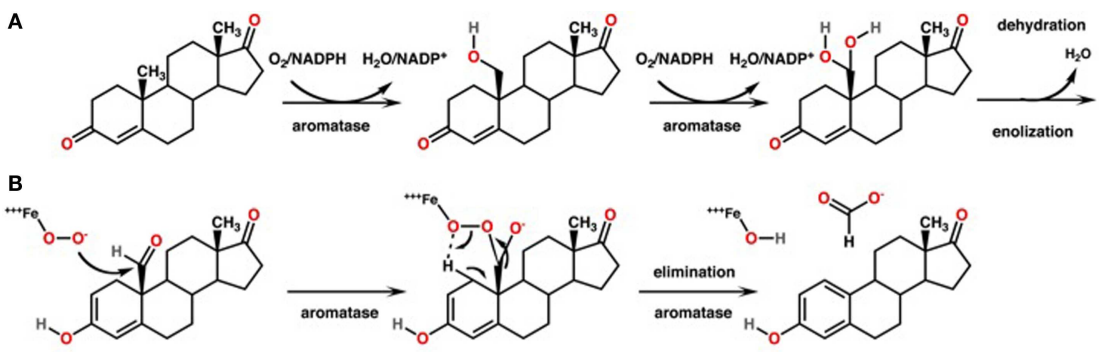

FIGURE 2 | Biosynthesis of estrgoens by aromatase. (A) General reaction for the conversion of testosterone to estradiol catalyzed by aromatase. **(B)** Catalytic mechanism of aromatase. The methyl group is a oxidized and subsequently eliminated.

density in reticular, lateral, and anterior-ventral thalamic nuclei (**Figure 3**). Very high levels were also found in the paraventricular hypothalamic nucleus.

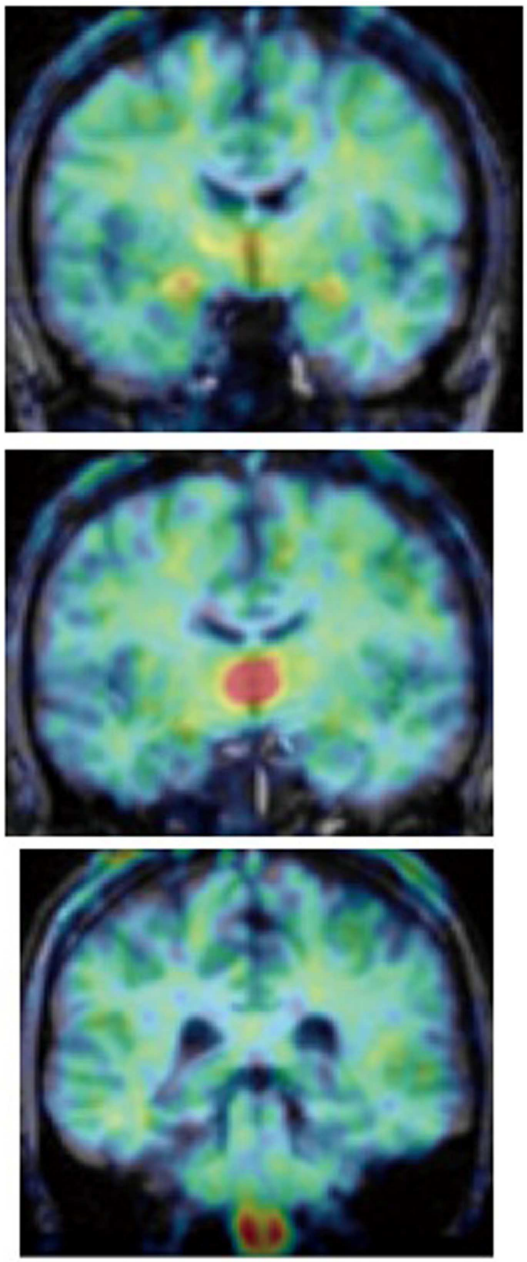

FIGURE 3 | Regional distribution of aromatase in the living human brain. Summed frames obtained from PET acquisitions over 90 min after [^{11}C]vorozole administration of a representative non-smoking subject were pseudocolored using the rainbow spectrum, such that red regions correspond with high density of radioactivity and blue corresponds to the lowest densities. The PET images are overlaid on a gray-level structural (T1 weighted) MRI scan of the same subject. From left to right: coronal slice at the level of amygdala and hypothalalmus; slice at the level of the medial thalamus and; slice at the level of the medulla (inferior olive; from Biegon et al., 2010b).

Moderately high levels of aromatase were noted in amygdala and preoptic area/anterior hypothalamus and in the medulla (inferior olive). Basal ganglia levels were relatively low, with visibly higher levels in the ventral striatum/nucleus accumbens (Biegon et al., 2010b). Labeled vorozole distributed to all cortical regions, with hippocampus indistinguishable from the temporal cortex. The distribution volume values derived from a two compartment model (Gunn et al., 2001; Logan, 2003; Logan et al., 2011) in both men and women (regardless of menstrual cycle) followed the rank order: thalamus > amygdala = preoptic area > medulla (inferior olive) > cortex = hippocampus, putamen, cerebellum, and white matter.

Earlier published postmortem studies were limited to a small number of preselected regions, although the combination of studies by different research groups and different methodologies support the notion that aromatase is ubiquitous in the human brain, although only a few regions express high levels of the enzyme (reviewed in Azcoitia et al., 2011 and Biegon et al., 2012).

To elaborate, aromatase *gene expression* was examined in postmortem samples from eight brain regions (Sasano et al., 1998). The amount of aromatase mRNA determined by RT-PCR assay in six cases (four men, two women) was highest in pons, thalamus, hypothalamus, and hippocampus. Analysis of multiple exons 1 revealed that exons 1f, considered specific for brain, as well as 1b (fibroblast type) and 1d (gonadal type), were expressed in the brain. Exons 1d and 1f tended to be utilized in hypothalamus, thalamus, and amygdala. The amount of overall mRNA expression was also higher in hypothalamus, thalamus, and amygdala than in other regions of the brain. There were no differences of utilization of exons 1 and mRNA expression of aromatase between female and male brain. The authors concluded that their results demonstrate that aromatase is expressed widely in human brain tissues in both men and women. The presence of aromatase transcripts in human temporal cortex, frontal cortex, and hippocampus was also confirmed by Stoffel-Wagner et al. (1999).

Aromatase *immunoreactivity* was found in hypothalamus, amygdala, preoptic area, and (cholinergic) ventral forebrain nuclei (Ishunina et al., 2005) in humans. Additional studies confirmed aromatase immunoreactivity in temporal cortex, hippocampus, and prefrontal cortex (Yague et al., 2006, 2010). Immunohistochemistry was also used to examine the cellular and subcellular distribution of aromatase in the human brain, establishing the presence of aromatase immunoreactivity in neurons as well as in glia. Thus, cortical and hippocampal aromatase was detected in pyramidal cells, granule cells, and interneurons; in perikarya, dendrites, axons, and axon terminals (Naftolin et al., 1996; Yague et al., 2006, 2010). The presence of glial aromatase was confirmed in prefrontal cortex, temporal cortex, and hippocampus, where it was associated with astrocytes (Yague et al., 2006, 2010).

Aromatase enzymatic activity was first described in the fetal human limbic system by Naftolin et al. (1971), followed by reports on activity in the adult brain and temporal cortex (Naftolin et al., 1996; Steckelbroeck et al., 1999). In agreement with the results from other methods described above, there were no differences in aromatase enzymatic activity between men and women and no

significant effect of aging in these brain regions (Steckelbroeck et al., 1999).

NICOTINE ADMINISTERED *IN VIVO* INHIBITS AROMATASE IN THE BRAIN

Nicotine modulation of brain aromatase *in vivo* was first reported by von Ziegler et al. (1991) in fetal and neonatal mice. After 1 or 2 weeks of prenatal exposure to 6 mg/kg nicotine delivered by an osmotic minipump, aromatase activity in male forebrains was significantly decreased at postnatal day 6, with no significant effects in females. The authors initially reported that nicotine alters brain aromatase activity only on postnatal day 6, the day when normal females show lower levels than normal males (von Ziegler et al., 1991). In subsequent studies in rats, it was shown that both nicotine and cotinine inhibited aromatase activity in the basal forebrain of male fetuses. Nicotine was twice as effective as cotinine and the effects of the two drugs were additive (Sarasin et al., 2003).

Using [^{11}C]vorozole, we have recently shown that acute *in vivo* exposure to nicotine doses which produced plasma levels similar to those found in smokers, resulted in significant region- and dose-dependent decreases in aromatase availability in the female baboon brain. The largest and most significant inhibition was found in the amygdala, where intravenous injection of 0.03 mg/kg nicotine reduced brain aromatase availability by ∼50% (Biegon et al., 2010b; **Figure 4**).

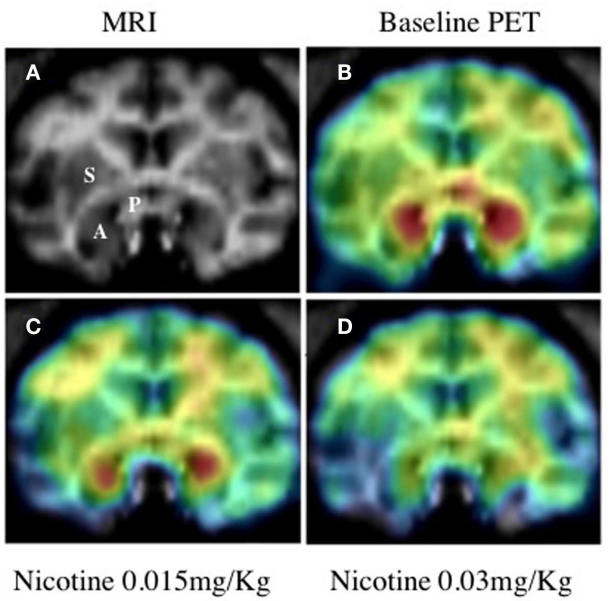

FIGURE 4 | Effect of nicotine on brain aromatase availability in the female baboon. Top: **(A)** Baboon brain MRI, coronal section at the level of amygdala. S, striatum; P, preoptic area; A, amygdala. **(B)** Representative baseline PET image coregistered with MRI. **(C)** PET image of same baboon following low dose nicotine (0.015 mg/kg). **(D)** PET image following injection of high dose (0.03 mg/kg) nicotine. PET images show averaged frames acquired between 52.5 and 90 min after tracer injection, pseudocolored using the rainbow spectrum, with purple indicating the lowest density and red indicating the highest density of radioactivity (from Biegon et al., 2010a).

BRAIN EFFECTS OF NICOTINE: SIMILARITIES AND DIFFERENCES WITH AROMATASE INHIBITION

The discovery of the ability of neuroendocrine tissues to aromatize androgens to estrogens was crucial to the formulation of the aromatization hypothesis (Naftolin and Ryan, 1975; McEwen et al., 1977), stating that testosterone synthesized by the fetal testis diffuses into the male brain where it is locally aromatized to estradiol and then initiates the process of masculinization, resulting, in adults, in the capacity to express male-typical sexual behaviors and high levels of aggression. In females, the aromatization process affects such functions as mood and appetite.

Consequently, aromatase inhibition can have a variety of sexually dimorphic effects on multiple domains including sexual functioning, mood, and cognition but the nature and persistence of the effects can vary greatly depending on the developmental status during exposure. The sections below provide a review of the brain effects of nicotine and aromatase inhibition. We highlight developmental, "organizational" brain effects (i.e., effects of maternal smoking during pregnancy) as well as effects of exposure in adolescents and adults when available in the literature, with the best supported similarities and differences summarized in **Table 1**.

SEXUAL BEHAVIOR

Prenatal nicotine exposure was shown to decrease male sexual behavior and to demasculinize male offspring (Segarra and Strand, 1989), paralleling the effects in aromatase "knock-out" (ArKO), and prenatal treatment with AI. In adult males, acute nicotine administration resulted in decreased intromission frequency, though this occurred only at 1.6 mg/kg, the highest dose tested (Retana-Marquez et al., 1993). Decreased sexual performance was also self reported in men exposed to cigarette smoke (Weisberg, 1985).

Studies (reviewed in Roselli et al., 2009) confirmed that male copulation is severely impaired in ArKO domestic mice in which the aromatase gene was selectively inactivated, consistent with the role for aromatization in both the organizational and activational effects of testosterone. Testosterone administration did not improve male sexual behavior in castrated ArKO adults, whereas

Table 1 | Similarities and differences in brain effects of nicotine and aromatase inhibition.

	Male		Female	
	Nicotine	**AI**	**Nicotine**	**AI**
Sexual behavior				
Prenatal exposure	Decrease	Decrease	Increase	Increase
Adult exposure	Decrease	?	Decrease	Decrease
Anxiety/Depression				
Prenatal exposure	No effect	No effect	Increase	Increase
Adult exposure	No effect	No effect	Decrease	Increase
Hot flashes			Increase	Increase
Weight gain	Decrease	Decrease	Decrease	Increase

AI, aromatase inhibition. Nicotine refers to smoking as well as nicotine alone. ?, there is not enough data on males to justify an increase or decrease effect.

combined treatment with estradiol and dihydrotestosterone (a non-aromatizable androgen) almost completely restored copulation behavior to levels observed in wild-type males. These results suggest that estrogens derived from aromatization of testosterone exert major activation effects on coital behavior in male C57Bl6 mice. The extent to which testosterone in men acts through aromatization to estradiol, is not yet clear. In eugonadal men, the estrogen receptor antagonist, tamoxifen, and the aromatase inhibitor, testolactone, had no adverse sexual effects. Furthermore, dihydrotestosterone was as effective as testosterone in maintaining sexuality in hypogonadal men, suggesting that aromatase was not involved (Gooren, 1985). The comparison of two men with congenital aromatase deficiencies, one with accompanying hypogonadism, suggested that testosterone alone allows for a normal sexual activity, but that there is a synergistic effect between testosterone and estradiol derived from aromatization. These findings suggest that aromatization may be required in men for completely normal sexual behavior, but that androgens are the main steroids involved.

In human females, prenatal exposure to nicotine resulted in a significant increase in same-sex orientation among female offspring (Ellis and Cole-Harding, 2001). The influence of aromatization on female sexual behavior is less clear but studies in female rats show that sexual behavior was enhanced by prenatal inhibition of androgen aromatization (Clemens and Gladue, 1978). In humans, gender role and sexual behavior are disrupted in girls with congenital adrenocortical hyperplasia, which increases testosterone levels (see recent review by Barenbaum and Beltz, 2011). Acute effects show that treatment in adult female rats increased lordosis in estrogen-treated ovariectomized female rats (Fuxe et al., 1977; Weaver and Clemens, 1987), but acute exposure to nicotine in adult men and women significantly reduced sexual arousal (Harte and Meston, 2008a,b). In addition, adult women given aromatase inhibitors report a loss of libido (Mitwally and Casper, 2003; Zivian and Salgado, 2008). These few studies on the sexual behavior of females may not be sufficient to establish a solid hypothesis on the direction of effects.

AGGRESSION

Prenatal smoking exposure and high trait aggression are associated variables in numerous animal and human studies, particularly during adolescence (Escobedo et al., 1997). This effect has been replicated in epidemiological studies and after controlling for important variables of heritability as mother's antisocial behaviors (Moffitt et al., 2008; Wakschlag et al., 2010). The observation that tobacco smoke exposure inhibits brain monoamine oxidase A (MAO A; Fowler et al., 1996), has led to the suggestion that the mechanism responsible for aggressive behavior in the offspring of females exposed to cigarette smoke during pregnancy is MAO A inhibition (Wakschlag et al., 2010). Indeed brain MAO A activity predicts trait aggression at adulthood (Alia-Klein et al., 2008, 2009). An additional and perhaps interactive mechanism in the effects of smoking on aggression is the inhibition of aromatase and little is known about the interactive potential of chronic MAO and aromatase inhibition.

Prenatal and developmental effects of smoking on aggression appear to be different than the acute effects of smoking in adults. In adults, smoking appears to reduce irritability and aggression. Furthermore, nicotine replacement therapy was found useful in reduction of agitation and aggression in smokers with schizophrenia (Allen et al., 2011).

Importantly, although numerous preclinical and clinical studies have shown that acute nicotine treatment reduces aggression, smoking deprivation results in negative mood, aggression, and hostility in adult rodents and humans (Schechter, 1974; Cherek, 1981). In a recent study, a lifetime history of cigarette smoking was associated with high traits of aggression and impulsivity in healthy and personality disorder participants and these effects may have started during prenatal development (Dakwar et al., 2011).

The effects on females appear to be similar to males in that smokers of both sexes exhibit negative emotionality as compared to non-smokers; however, the data is suggestive that smoking affects aggression in males more than in females, where it has a primary effect on depression (Pogun and Yararbas, 2009).

Aggressive behavior, long thought to be controlled by testosterone, also appears to be strongly dependent on aromatization and estrogen in both mammalian and non-mammalian species (see review by Trainor et al., 2006). Estrogen has been shown to modulate aggression in a variety of species. Although in most cases estrogen increases the probability and intensity of male-on-male aggressive behavior, there are exceptions in which estrogen decreases the intensity of aggression. Thus, the duration of aggressive behavior in resident-intruder tests was extremely low for ArKO mice compared to wild-type mice and estradiol injections restored aggression to wild-type levels. However, when the production of estrogen was blocked by an AI (fadrozole) in California mice, the males were more aggressive compared to controls, indicating that production of estrogen is associated with reduced aggression in California mice.

DEPRESSION AND ANXIETY

Developmental effects of prenatal smoking exposure increase the risk for depression particularly in females. Female offspring of rodents prenatally treated with nicotine show vulnerability during adolescence to depression and early smoking onset; more so than males (Romero and Chen, 2004; Vaglenova et al., 2004). Findings from human and animal studies infer sex and region specific effects and suggest a role for smoking in higher rates of depression, especially among adolescent females (Kandel et al., 1994; Cornelius and Day, 2009). Smoking during pregnancy has psychological effects on the mothers as well as their offspring. In a study of persistent pregnant smokers, the smokers reported higher prenatal stress and depression than non-persistent smokers or non-smokers (Eiden et al., 2011). Women were also found to be more vulnerable to the depressive symptoms of nicotine withdrawal (Gaffin et al., 2011). Acutely, nicotine has been found to be more anxiolytic in female than in male rats (Harrod et al., 2004), corroborating human studies which also document that females are more susceptible to the effects of nicotine on anxiety (Pogun and Yararbas, 2009).

In males nicotine has been shown to produce antidepressant-like responses in rats subjected to the forced swimming test, a popular model of depression in rodents. While this effect is not expected to reflect inhibition of aromatase, it is relevant to note that orchiectomy abolished the antidepressant effect of nicotine and its restoration required supplementation with estradiol (Bonilla-Jaime et al., 2010).

Decreased aromatase availability appears to have sexually dimorphic effects on depression and anxiety in rodents. ArKO females displayed decreased active behaviors, such as struggling and swimming, and increased passive behaviors, such as floating, in repeated sessions of the forced swim test, indicating that these females exhibit "depressive-like" symptoms (Dalla et al., 2004). This effect was not observed in males (Dalla et al., 2005). By contrast, ArKO males did not differ from WT in spontaneous motor activity, exploration, or anxiety. These findings are in line with the absence of major neurochemical alterations in hypothalamus, prefrontal cortex, or striatum, which are involved in the expression of these behaviors.

Clinical and community studies of women have shown that both anxiety and depression are increased in women taking aromatase inhibitors for breast cancer (Mitwally and Casper, 2003; Zivian and Salgado, 2008). Long-term efficacy and safety of the use of AI in men and boys has not been established to date, although sex differences in brain-related effects are likely due to the inherently different implications of changing the estrogen/androgen ratio in males and females and also because higher levels of testosterone in males compared to females renders it more difficult to effectively inhibit aromatase in men (de Ronde and de Jong, 2011).

APPETITE

Prenatal exposure to nicotine appear to alter saccharin preferences in a sexually dimorphic manner, eliminating an observation in untreated animals where females had a higher preference to saccharin than untreated males. In the animals exposed to nicotine prenatally, there was an increase in males preferring saccharin to the female level (Lichtensteiger and Schlumpf, 1985). This study provides another example of a demasculinizing effect of prenatal nicotine as would be expected to result from prenatal aromatase inhibition.

The situation is different in adults, where acute nicotine administration is considered to be a powerful appetite suppressant. In fact, the effects of smoking on body weight are a concern since more women than men report smoking to avoid weight gain (Pomerleau and Kurth, 1996). This effect is at least partly biologically based since studies of Sprague-Dawley rats showed greater effects of nicotine on food intake and body weight in female than male rats (Grunberg, 1986). The appetite-suppressing effect of nicotine appears to be mediated through direct interaction with neurons involved in initiation of feeding behavior (Mineur et al., 2011) and is not likely to be mediated through aromatase inhibition, though other effects of nicotine related to appetite may rely on this mechanism. Thus, nicotine appears to increase the reinforcing properties of food (reviewed in Donny et al., 2011) and older studies report increased consumption of sweet foods following nicotine deprivation (Hughes et al., 1991; Spring et al., 2003).

Estrogen is known to suppress feeding and weight gain in mammalian females including women. An important contribution to this effect of estrogen is suppression of feeding by direct action with brain centers engaged in appetite control (Nunez et al., 1980; Butera and Beikirch, 1989; Dagnault and Richard, 1997; reviewed in Geary, 2004). Consequently, inhibition of aromatase is expected to increase feeding and weight gain, as reported by Kubatka et al. (2008a,b) who found increased weight gain in female rats treated with two different aromatase inhibitors – anastrozole and letrozole. Increased weight gain was reported by close to 50% of a community sample of 1200 women taking aromatase inhibitors for breast cancer (Zivian and Salgado, 2008). In the same vein, we found a negative correlation between aromatase in the amygdala and BMI, which was more pronounced in women than in men (Wang et al., 2011). However it is noteworthy that loss of appetite is also reported by some women receiving aromatase inhibitors (Mitwally and Casper, 2003) amounting to 8% of responders in a community sample (Zivian and Salgado, 2008). There appear to be important sex differences in the control of food intake (Geary and Lovejoy, 2008). Interestingly, in male rodents, peripubertal inhibition of aromatase leads to a decrease rather than an increase in body growth and weight gain (Bajpai et al., 2010).

HOT FLASHES

Current smoking (and high BMI), reportedly predispose women to more severe or frequent hot flashes (Whiteman et al., 2003). In perimenopausal women ages 45–54, current smokers had significantly higher androstenedione levels and a higher androgen to estrogen ratio than never smokers. Current smokers also had lower progesterone levels and increased odds of experiencing hot flushes compared to never smokers (Cochran et al., 2008). This effects is sex specific since there are no reports on hot flushes in healthy male smokers. It is noteworthy that men treated for prostate cancer with androgen ablation therapy (e.g., by injections of gonadotropin-releasing hormone analogs) report daily hot flashes, which respond to estrogen treatment (Gerber et al., 2000).

Hot flashes are the most common adverse effect reported in clinical trials and community studies of aromatase inhibitors in women, with up to 75% of users reporting this adverse effect (Bonneterre et al., 2000; Mitwally and Casper, 2003; Howell et al., 2005; Zivian and Salgado, 2008). Hot flashes arise when the thermoregulatory centers in the hypothalalmus are deprived of estrogen (see review by Rossmanith and Ruebberdt, 2009) and are a classical symptom of estrogen deprivation resulting from menopause and ovariectomy as well as the most common side effect of estrogen receptor antagonists and selective estrogen receptor modulators like tamoxifen (e.g., Bonneterre et al., 2000; Howell et al., 2005).

COGNITION AND NEUROPROTECTION

Prenatal exposure to nicotine impairs cognitive functions, such as memory and attention, and this impairment is gender-specific. Deleterious effects in male but not female offspring were documented on synaptic function, cell signaling, and cell number. In terms of cognitive performance, marked deficits were observed in males in auditory attention (Niaura et al., 2001; Cornelius and Day, 2009). Other studies show that on average, male smokers perform more poorly than female smokers on attention and memory tasks (Abreu-Villaca et al., 2003; Jacobsen et al., 2007).

In females, stimulation of estrogen receptors, which enhances hippocampal neurogenesis and synaptic plasticity, may be protective through providing greater adaptive capacity (Tanapat et al., 1999; McEwen, 2002). However, this protection relative to males is diminished in prenatally exposed rats who were treated with

nicotine later in adolescence (Slotkin et al., 2007). Human studies also documented that nicotine exposed females showed reduced performance accuracy in neuropsychological tasks (Niaura et al., 2001; Cornelius and Day, 2009).

A meta analysis of the acute effects of nicotine and smoking on adult human performance found significant positive effects of nicotine or smoking in several domains, including short-term and working memory, with effect sizes ranging from 0.16 to 0.44 (Heishman et al., 2010). Treatment with nicotine receptor agonists has been shown to elicit improvement of cognitive performance in a variety of behavioral tests in rats, monkeys, and humans (Reviewed in Mudo et al., 2007).

Evidence for the involvement of aromatase in support of cognitive function and neuroprotection in various species was recently reviewed by Garcia-Segura (2008) and Roselli et al. (2009). More recently, AI were found to inhibit hippocampal learning (long-term potentiation, LTP) and dendritic spine formation (Zhou et al., 2010; Vierk et al., 2012) although the effects on LTP appear to be sexually dimorphic, with significant effects found in female but not male mice. In humans, a testosterone-supplementation study in elderly men showed that the addition of an AI reversed the beneficial effects of testosterone on verbal memory with no effect on spatial memory (Cherrier et al., 2005). A similar study in women did not reveal statistically significant effects of AI, although the authors suggest the small size of the study may be a contributing factor (Shah et al., 2006).

In preliminary studies at our laboratory, availability of aromatase in the amygdala was negatively correlated with performance on verbal learning and memory tests in men, while in women, aromatase in the amygdala was positively correlated with high scores on trait constraint (Biegon et al., 2011a,b). Thus, although there were no sex differences in aromatase availability in amygdala or learning and memory scores nor traits in our sample, the amygdala aromatase availability was associated with sex specific effects on verbal learning, memory, and personality traits.

ALZHEIMER'S DISEASE

Acute nicotine injections and chronic treatment with nicotine patch have been found to improve attentional performance in patients with Alzheimer's Disease (AD; White and Levin, 1999). The neural substrate for nicotine-induced improvement in attention is hypothesized to be related to the basal forebrain cholinergic system (Lawrence and Sahakian, 1998).

Epidemiological studies initially indicating a lower incidence of AD in smokers now suggest conflicting results. Clinical and pathology findings also are mixed as to how smoking behavior affects the manifestation of AD markers and the relevance of aromatase inhibition is unclear. Studies that show nicotine-induced increases in nAChR and protection against age-related nAChR decline, contrast perhaps in a functionally relevant way, to losses of nAChR in AD. Although epidemiological, clinicopathological, and functional studies in humans do not present a cohesive picture, much *in vitro* data suggest neuroprotective properties of nicotine when used in models of neurodegenerative disorders. Studies of nicotine and nicotinic agonist effects on cognitive function in the non-demented and in AD are not compelling. More work is needed to ascertain whether acute or repetitive activation of nAChR with acute or intermittent exposure to nicotine or the persistent inactivation of nAChR with chronic nicotine exposure is a therapeutic objective and/or explains any pro-cognitive effects of those drugs. Other studies show complex interactions between nAChR, nicotinic agonists, and agents implicated in AD etiology (reviewed in Sabbagh et al., 2002).

Aromatase has been suggested to play a role in AD (for review see Hiltunen et al., 2006) although available data are not sufficient to establish a neuroprotective effect. Human genetic studies have revealed evidence of a relationship between mutations of the aromatase gene and AD risk. Subjects who had single nucleotide polymorphisms (SNPs) in the CYP19 gene were reported to have an ∼60% increase in the risk for AD (Iivonen et al., 2004). Likewise, a twofold increased risk for AD was observed in ApoE ε4 carriers who had SNPs in the CYP19 gene (Huang and Poduslo, 2006). Studies on postmortem brains revealed enhanced aromatase expression in the nucleus basalis of Meynert and decreased aromatase expression in the hypothalamus of AD patients, with no sex differences observed in any of the nuclei studied (Ishunina et al., 2005). In contrast, in hippocampus samples from women, estrogen receptors (ERα subtype), and aromatase expression were found to increase with age, and to decrease in AD (Ishunina et al., 2007). An earlier study on frontal and temporal cortical samples did not find significant difference in aromatase activity between AD brains and controls (Wozniak et al., 1998).

ADDICTION

The rates of cigarette smoking have declined over the last few decades, but smoking decreases among women are less pronounced (Mathers and Loncar, 2006) and smoking rates among girls and young women are increasing (CASA investigators, 2006). Paralleling animal studies, human females appear to become addicted faster than men, while finding it harder to quit (Bohadana et al., 2003). In offspring of women who smoked during pregnancy, the odds of progressing to nicotine dependence were almost doubled compared to offspring from non-smoking women (Cornelius and Day, 2009). Furthermore, nicotine replacement in the context of smoking cessation appears to be less effective in women (Bohadana et al., 2003; CASA investigators, 2006). A question remains how or through what mechanism might cigarette smoking become a gateway to smoking addiction and to other drugs. Another question is how observed sex differences in smoking age of onset and rates of smoking cessation may be related to the actions of aromatase inhibition by smoking.

There is no evidence that aromatase inhibitors are addictive, in stark contrast to the potent addictive properties of nictoine and smoking (Nusbaum et al., 2000; Bohadana et al., 2003). However, several lines of evidence suggest that aromatase may modulate various aspects of nicotine addiction in a sex specific manner. Addiction to other substances is also impacted. Female animals consume more alcohol than males and interestingly, this effect is reversible by gonadectomy (Almeida et al., 1998). This effect is not restricted to alcohol but is also observed with psychostimulants as cocaine.

Sex differences are also found in the context of relapse following periods of abstinence, where as with cigarette smoking, females find it harder to quit than males.

Cigarette smoking is very common among drug addicted individuals, and it appears that methadone, used to facilitate treatment of opioid addiction, is metabolized by aromatase and may act as a potent inhibitor of aromatase *in vivo*. These findings may contribute to variability in methadone clearance, to drug–drug interactions, and to side effects observed in male and female patients. Because methadone seems to be able to inhibit aromatase, it follows that methadone may alter the metabolism and disposition of endogenous testosterone and androstenedione. Lower concentrations of estradiol have been documented in men taking methadone (Hallinan et al., 2009), and low bone mineral density that may be due to low estrogen concentrations has been documented in 83% of patients in a methadone maintenance treatment program (Kim et al., 2006). It is conceivable that other side effects of methadone, which include flushing, muscle pain, and symptoms reminiscent of estrogen withdrawal (Senay, 1985; Backstrom, 1995), may be explained in part by the drug's action on aromatase.

DISCUSSION AND CONCLUSION

As shown above, aromatase and its activity in the brain can be parsed to basic psychological processes; effects are observed in affect, learning, and memory, libido, and appetite; and sex differences in the direction and size of the effects are very common. The effects of smoking can also be parsed to the same psychological processes, and sex differences in nicotine action have been recognized in animals as well as human subjects (e.g., recent review by Pogun and Yararbas, 2009).

This literature review highlights findings from animals and humans indicating that smoking and nicotine target brain functions which are also shaped and influenced by aromatase activity. While it has been extensively documented that nicotine interacts with the nicotinic acetylcholine receptor as its mechanism of action, this does not exclude additional mechanisms contributing to the effects of smoking and nicotine on aggression, cognitive function, anxiety, depression, sexual drive, and appetite (e.g., Fowler et al., 1996).

Available findings summarized above highlight the possibility that several of the known effects of nicotine, most prominently effects on sexual and depression-like behavior and induction of hot flashes, may be mediated or modulated by changes in aromatase activity (**Table 1**) in an age- and sex-dependent manner. It is noteworthy that these effects of nicotine are more prominent in periods such as prenatal development, adolescence and the perimenopausal period, which are associated with relatively abrupt changes in estrogen synthesis (a prenatal peak in brain aromatase in males, a peripubertal increase in ovarian production in girls and a perimenopausal decrease in oarain production in adult women). Such a profile is to be expected from a partial rather than complete inhibition of aromatase activity exerted by nictoine and similar compounds which act as competitive inhibitors of aromatase with low to moderate affinity (Barbieri et al., 1986; Biegon et al., 2010a). Thus, we have shown that nicotine doses producing plasma levels comparable with those found in smokers are capable of a significant but partial (∼50%) inhibition of aromatase. Obviously, more research is indicated to substantiate and better understand the effects of aromatase inhibition on brain function in this context. However, the notion that aromatase inhibition by nicotine and other tobacco alkaloids is an alternative/additional mechanism for the effects of cigarette smoking on human behavior, provides a mechanistic explanation to observed sex differences in smoking and a possible source of new treatment and prevention approaches for the initiation, physiological and psychological consequences, and for the cessation of smoking.

FUTURE PERSPECTIVES

While research on the brain effects and mechanisms of nicotine action has been ongoing for many decades, specific AIs are relatively new agents. Furthermore, despite the fact that several AIs are approved drugs used in the clinical setting, the foremost clinical use of these agents is in the adjuvant treatment of breast cancer (e.g., Buzdar and Howell, 2001), and clinical studies of breast cancer do not include systematic investigation of neuropsychological effects. Consequently, there is a great need for additional basic research on the effects of aromatase inhibition in males and females across the life span. Such studies, as well as side-by-side comparisons of aromatase inhibitors and nicotine and other tobacco constituents have the potential to shed light on sexually dimorphic effects in brain function and structure and in important functioning domains such as mood, learning, and memory. Since smoking addiction is replete with sex differences, future exploration of sex specific, pharmacological interventions targeting specific androgen, or estrogen receptors for prevention or treatment is also warranted.

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