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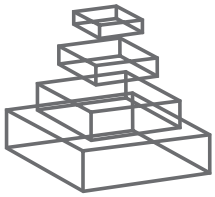

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NEURONAL CELL SIGNALING AND BEHAVIOR

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Learning and memory functions as well as many neurodegenerative and neuropsychiatric disorders, including Parkinson's disease, drug addiction and schizophrenia are caused by dysregulation of cell signaling mechanisms in the brain.

This issue of Frontiers will provide evidence for signal transduction alterations implicated in cognitive and non-cognitive behaviors, as investigated by means of pharmacological and genetic approaches. Specialists in the field will be invited to contribute articles covering the impact on behavior of manipulations of neurotransmitter systems, intracellular signaling cascades and gene expression.

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EDITORIAL
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Neuronal signaling and behavior

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The last years have witnessed a number of important technical breakthroughs, which have improved considerably the ability to investigate the contribution of molecular processes to physiological and pathological conditions. This is particularly evident with regard to the study of the involvement of specific signal transduction mechanisms in brain function and disease. Increasingly sophisticated transgenic approaches combined with the use of a vast array of behavioral models have led to a profound and detailed view of the way in which distinct brain regions and neuronal populations are affected by environmental and pathological challenges.

The scope of the present volume is to provide a panoramic view of recent progress in this direction, with special emphasis on key molecular mechanisms implicated in learning and memory function, as well as in neurodegenerative and neuropsychiatric disorders, including Parkinson's disease, epilepsy, schizophrenia, and drug addiction.

In the first Original Article, Penke et al. (2011) describe the existence of distinct mechanisms at the basis of the regulation, in the hippocampus, of *Arc/Arg3.1*, an immediate early gene implicated in synaptic plasticity. They show that, during spatial exploration, early expression of *Arc/Arg3.1* occurs independently of the transcription factor *Egr1/Zif268*. In contrast, *Egr1/Zif268* is necessary for a late, prolonged wave of *Arc/Arg3.1* expression in response to electroconvulsive shock.

Signaling in epileptic behavior is the subject of the Review Article by Bozzi et al. (2011). Here, the authors concentrate on the pathways activated by modulatory neurotransmitters (e.g., dopamine, norepinephrine, and serotonin), which lead to changes in gene expression during acute seizure events. They also discuss the deleterious consequences of seizure activity, focusing on the contribution of specific signaling pathways to the progression of the disease.

The Original Article by Pozzi et al. (2011) provides evidence indicating that activation of the cAMP response element-binding protein (CREB), at different locations within the corticostriatal circuitry, is associated to distinct behavioral responses involved in visuospatial attention.

Several Review Articles are centered on specific molecular mechanisms implicated in memory formation and cognition. Sindreu and Storm (2011) provide a timely and exhaustive overview of the importance of zinc homeostasis and zinc-activated signal transduction pathways [such as the extracellular signal-regulated kinase (ERK) cascade], in synaptic plasticity and memory, with special focus on the relevance of these mechanisms in the hippocampus and amygdala. On the same line, a comprehensive Review Article by Fasano and Brambilla (2011) deals with the implication of abnormal Ras-ERK signaling in drug addiction, intellectual disability and L-DOPA induced dyskinesia, a severe and frequent motor disorder affecting Parkinson's disease patients.

Two Original Articles by d'Isa et al. (2011) and Silingardi et al. (2011), discuss the role of Ras-GRF1, a neuronal specific activator of Ras, in various forms of memory. Interestingly, Ras-GRF1 seems to have a complex role, being dispensable for most forms of declarative learning (but not object recognition), while playing a crucial role in emotional memories.

In their Review Article, Gal-Ben-Ari and Rosenblum (2012) discuss the behavioral paradigms to study the sense of taste with regard to learning, memory, and consolidation. They also present a comprehensive overview of the neuronal structures, the neurotransmitter systems, and the signaling components involved in these processes.

The application of transgenic techniques to link distinct neuronal populations and signal transduction pathways to specific behaviors has been particularly useful in the study of the basal ganglia, a group of brain structures affected by numerous neurodegenerative and neuropsychiatric diseases.

The Review Article by Ena et al. (2011) describes the contribution of new methodologies, such as bacterial artificial chromosome (BAC) transgenesis, optogenetic, and viral transgenesis, to the study of the striatum, which is the major component of the basal ganglia. The use of cell-specific ablation and conditional knock-out is discussed with regard to the characterization of the role played by distinct populations of striatal neurons in motor behavior and in motivational processes implicated in drug addiction.

Basal ganglia transmission and striatal neurons is also the main topic of the Review Article by Feyder et al. (2011), which provides an update of the mechanisms implicated in L-DOPA-induced dyskinesia, with particular focus on abnormal signaling processes affecting the cAMP and ERK cascades and occurring in dopamine D1 receptor expressing striatal neurons.

The dopamine- and cAMP-regulated phosphoprotein Mr 32,000, DARPP-32, is a key signaling molecule necessary for a correct functioning of the striatum and basal ganglia. Yger and Girault (2011) offer a critical appraisal of the molecular properties of this important signaling component, focusing on the regulation by various classes of psychoactive drugs and on the involvement in neuropsychiatric diseases.

The mammalian target of rapamycin (mTOR), is a fundamental regulator of mRNA translation and has been implicated in a plethora of physiological and pathological conditions. This subject is addressed by Santini and Klann (2011) who describe the molecular mechanisms at the basis of mTOR-mediated control of *de novo* protein synthesis and provide a number of examples of how perturbations of this signaling cascade represent a common pathophysiological feature of neurodevelopmental, neuropsychiatric, and neurodegenerative disorders.

We hope that the reader will enjoy this collection of articles and find it useful as a source of information on a number of specific aspects related to a broad subject of study. We are very grateful to the many contributors and to all the colleagues involved in the reviewing process for having made this endeavor possible.

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REVIEW ARTICLE
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Unraveling the differential functions and regulation of striatal neuron sub-populations in motor control, reward, and motivational processes

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The striatum, the major input structure of the basal ganglia, is critically involved in motor control and learning of habits and skills, and is also involved in motivational and reward processes. The dorsal striatum, caudate–putamen, is primarily implicated in motor functions whereas the ventral striatum, the nucleus accumbens, is essential for motivation and drug reinforcement. Severe basal ganglia dysfunction occurs in movement disorders as Parkinson's and Huntington's disease, and in psychiatric disorders such as schizophrenia and drug addiction. The striatum is essentially composed of GABAergic medium-sized spiny neurons (MSNs) that are output neurons giving rise to the so-called direct and indirect pathways and are targets of the cerebral cortex and mesencephalic dopaminergic neurons. Although the involvement of striatal sub-areas in motor control and motivation has been thoroughly characterized, major issues remained concerning the specific and respective functions of the two MSNs sub-populations, D_2R-striatopallidal (dopamine D_2 receptor-positive) and D_1R-striatonigral (dopamine D_1 receptor-positive) neurons, as well as their specific regulation. Here, we review recent advances that gave new insight in the understanding of the differential roles of striatopallidal and striatonigral neurons in the basal ganglia circuit. We discuss innovative techniques developed in the last decade which allowed a much precise evaluation of molecular pathways implicated in motivational processes and functional roles of striatopallidal and striatonigral neurons in motor control and in the establishment of reward-associated behavior.

Keywords: striatum, medium-sized spiny neurons, transgenic mouse model

INTRODUCTION

The basal ganglia are composed of several interconnected nuclei involved in adaptive control of motor, cognitive, and motivational behavior. Dysfunctions of the basal ganglia system occur in several neuro-psychiatric diseases as Parkinson's (PD) and Huntington's diseases, addiction, attention deficit hyperactivity disorder (ADHD), schizophrenia, and Tourette's syndrome (Delong, 1990; Comings, 2001; Saka and Graybiel, 2003; Chao and Nestler, 2004; Graybiel, 2005; Delong and Wichmann, 2007; Keshavan et al., 2008). The striatum, the main input structure of this system, is subdivided in a dorsal part mainly involved in motor control (Graybiel et al., 1994; Hikosaka et al., 2000; Packard and Knowlton, 2002; Yin and Knowlton, 2006; Nicola, 2007) and the ventral striatum or nucleus accumbens (NAc) which is implicated in motivational and reward processes (Belin et al., 2009). In rodents, the striatum is composed of about 95% of GABAergic projection medium-sized spiny neurons (MSNs) and 5% of interneurons, including three subtypes of GABAergic neurons and the large aspiny cholinergic neurons (Kawaguchi et al., 1995; Bolam et al., 2000; Tepper and Bolam, 2004). MSNs can be subdivided in two neuronal sub-populations according to their projection sites and their expression in receptors and neuropeptides (Graybiel, 2000). Striatonigral neurons, giving rise to the direct pathway, project monosynaptically to the substantia nigra *pars reticulata* (SNr) and the medial globus pallidus (MGP), the output structures of basal ganglia. They are enriched in the neuropeptides substance P (*Tac1*) and dynorphin (*Pdyn*) and in dopamine D_1 receptor (D_1R; *Drd1a*) and M4 muscarinic acetylcholine receptor (*Chmr4*; Gerfen and Young, 1988; Gerfen et al., 1990; Le Moine et al., 1991; Bernard et al., 1992; Ince et al., 1997). Striatopallidal neurons participate to the indirect pathway and project to the lateral globus pallidus (LGP). This indirect pathway reaches the SNr/MGP by synaptic relay through the subthalamic nucleus (STN). Striatopallidal neurons specifically express the neuropeptide enkephalin (*Penk1)* and dopamine D_2 receptor (D_2R; *Drd2*) and adenosine A_{2A} receptor (A_{2A}R; *Adora2a*; Gerfen and Young, 1988; Gerfen et al., 1990; Schiffmann et al., 1991, 2007).

In the 90s, Albin et al. (1989) propose a model of the basal ganglia in which the direct and indirect pathways would have an opposite but balancing role in the control of the motor behavior. In this model, the striatonigral direct pathway would promote the movement whereas the striatopallidal indirect pathway would inhibit the motor behavior (Albin et al., 1989; Delong and Wichmann, 2007). MSNs are also involved in reward and motivational processes (addiction) but their differential function in the establishment of such behavior are still poorly understood.

The two MSN populations are morphologically very similar and heterogeneously distributed in the striatum making difficult a specific identification and analysis. These characteristics preclude a satisfactory demonstration of their differential functions in the motor and reward behavior for decades. The recent emergence of new

methodologies as BAC transgenesis, optogenetic, viral transgenesis allowing to target these neurons has given the opportunity to override this problem leading to a more specific investigation of striatopallidal and striatonigral neuron functions in the basal ganglia circuit. Here, we review the most recent advances regarding the differential role of striatopallidal and striatonigral neurons in the basal ganglia system. We will describe the innovative techniques used to investigate the gene expression profiles and the molecular pathways involved in the response to multiple stimuli (i.e., psychostimulants, 6-OHDA-induced dopamine depletion L-DOPA) in the two MSN sub-populations. We will also discuss the important contribution of cell-specific ablation models and conditional knock-out models to unravel the functional roles of these striatopallidal and striatonigral neurons in the motor and motivational behavior.

BAC TRANSGENIC REPORTER MICE AND EVALUATION OF MOLECULAR SIGNALING IN STRIATOPALLIDAL AND STRIATONIGRAL NEURONS

The recent development of transgenic BAC reporter mouse lines in which the enhanced green fluorescent protein (EGFP) gene is selectively expressed in a large variety of neuronal sub-populations represents a powerful tool for identifying each neuronal subtype in different experimental paradigms. More particularly, BAC EGFP transgenic mice allowed to specifically identify and study the differential functional *in vivo* signaling of the two distinct MSN neuronal populations. In these transgenic mice, EGFP or dtTomato (a red fluorescent protein) are expressed under the control of *Drd1a* or *Drd2* promoter in order to target the striatonigral (*Drd1a*–EGFP and *Drd1a*–dtTomato) and striatopallidal neurons (*Drd2*–EGFP), respectively (Gong et al., 2003; Shuen et al., 2008). The detailed study of the different mouse lines matched and confirmed previous *in situ* hybridization or immunohistochemical studies (Gerfen and Young, 1988; Le Moine et al., 1991; Schiffmann et al., 1991; Levesque et al., 2003) that led to the two-pathways model of basal ganglia. The *Drd1a*–EGFP and *Drd1a*–dtTomato mice, as well as A *Chrm4*-EGFP line, allowed the identification of striatonigral neurons and their projection structures (MGD and SNr) whereas the *Drd2*–EGFP labeled striatopallidal neurons and their projection area (LGP) but also the cholinergic interneurons as well as neurons in the substantia nigra *pars compacta* and ventral tegmental area corresponding to neurons expressing D_2R as an autoreceptor (Gong et al., 2003; Bertran-Gonzalez et al., 2008; Shuen et al., 2008; Matamales et al., 2009). Thus, these mice are valid tools to specifically identify the two MSN populations and allowed to investigate and compare molecular pathways and molecular changes taking place in striatopallidal and striatonigral neurons in response to different stimuli (Bertran-Gonzalez et al., 2008, 2009; Borgkvist et al., 2008; Santini et al., 2009).

The extracellular signal regulated kinase (ERK) cascade is an important signaling pathway that underlies synaptic plasticity, cellular excitability and learning. ERK is activated in the striatum by coordinated dopamine and glutamate receptor signaling, where it underlies corticostriatal synaptic plasticity and influences striatal cell excitability. ERK activation is necessary for action–outcome learning and performance of goal-directed actions (Shiflett and Balleine, 2010). The activation of the ERK pathway distinctly in the two MSN populations has been implicated in the long lasting effect of drugs of abuse (Valjent et al., 2006; Girault et al., 2007). Bertran-Gonzalez et al. (2008) demonstrated by immunofluorescence on *Drd2*–EGFP and *Drd1a*–EGFP mice that the ERK pathway activation occurs specifically in striatonigral neurons after acute and even repeated cocaine administration. Moreover, this activation is concomitant to the phosphorylation of MSK1, an important player in the phosphorylation of CREB and histone H3 in the striatum in response to cocaine (Bertran-Gonzalez et al., 2008). This ERK pathway is also functional in striatopallidal neurons. The antipsychotic drug haloperidol known to antagonize D_2R (Farde et al., 1988) induced an activation of ERK, MSK1, and histone H3 exclusively in striatopallidal neurons, showing the complete segregation between the striatopallidal and striatonigral signaling pathways within the striatum in response to diverse pharmacological stimuli (Bertran-Gonzalez et al., 2009). In contrast to striatonigral neurons, in striatopallidal neurons the H3 phosphorylation is occurring in an ERK–MSK1-independent manner (Bertran-Gonzalez et al., 2009). H3 phosphorylation involves the activation of the $A_{2A}R$-Golf signaling cascade leading to the DARPP-32 phosphorylation and the inhibition of PP-1. Thus, the pathways involved in the phosphorylation of the histone H3, reflecting the chromatin remodeling, are clearly different in striatopallidal and striatonigral neurons. Santini et al. (2009) have demonstrated that the same molecular mechanism observed in response to drug of abuse in striatonigral neurons could be implicated in L-DOPA-induced dyskinesia. Single injection of L-DOPA in a 6-OHDA mouse model induced ERK activation in association with an increase of MSK1 and histone H3 phosphorylation. Moreover, chronic administration of L-DOPA produced a persistent increase of ERK, phospho-MSK1 and phospho-H3 only in dyskinetic but not in non-dyskinetic mice and this increase is restricted to striatonigral neurons (Santini et al., 2009). Therefore, this increase in phosphorylated H3 could be associated with gene expression changes in striatonigral neurons that might play a role in the development of L-DOPA-induced dyskinesia.

Dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) is a protein phosphatase inhibitor highly expressed in striatal MSNs (Walaas et al., 1983). DARPP-32 is a key regulator of protein phosphatase (PP-1) and protein kinase (PKA) signaling. DARPP-32 can be phosphorylated on different threonine residues that modify its activation state and induce opposite biochemical effects (inhibition of protein phosphatase or protein kinase; Fisone et al., 2007). Therefore, by acting on the phosphorylation state of multiple downstream targets, DARPP-32 is an important actor in striatal neurons; in control of their electrophysiological behavior and their implication in behavioral responses as reward, motor learning, and L-DOPA-induced dyskinesia (Valjent et al., 2005; Fisone et al., 2007; Santini et al., 2009; Bateup et al., 2010). Considering this central role, the respective involvement of DARPP-32 in striatopallidal and striatonigral neurons functions was extensively studied. By using BAC transgenic mice expressing a tagged DARPP-32 specifically in striatopallidal or striatonigral neurons, Bateup et al. (2008) showed that the activation of D_1R or D_2R results in an opposite pattern of DARPP-32 phosphorylation. D_1R activation leads to an increase of T34 phosphorylation and a decrease of T75 phosphorylation whereas D_2R activation leads to the opposite effect. Moreover, different subclasses of psychostimulant and antipsychotic drugs are able to induce cell-specific pattern of

DARPP-32 phosphorylation. Whereas cocaine induces specific T34 phosphorylation in striatonigral neurons, haloperidol treatment induces the same phosphorylation exclusively in striatopallidal neurons (Bateup et al., 2008). Moreover, Stipanovich et al. (2008) have shown using *Drd1a*–EGFP mice that psychostimulant injection lead to the nuclear accumulation of phospho-T34-DARPP-32 in striatonigral neurons. They demonstrated that D_1R activation leads to the phosphorylation of DARPP-32 on serine 97 that is responsible for the nuclear export and accumulation of DARPP-32. This accumulation is also required for the phosphorylation of histone H3 and then is essential for regulation of gene expression. The knock-in mouse model bearing a point mutation of the serine 97 in alanine (S97A) showed that S97 mutation, and hence disruption of DARPP-32 nuclear accumulation, produced alteration in the long lasting effect of drug of abuse, and decreased motivation for food reward (Stipanovich et al., 2008). Meurers et al. (2009) have also recently shown in a striatopallidal and striatonigral neurons profile analysis that DARPP-32 pathway seems to be the target of adaptive modification in the two MSN populations in response to dopamine depletion and L-DOPA treatment.

Besides differences in signaling pathways, the study on *Drd1a*- and *Drd2*-EGFP mouse lines also allowed to firmly demonstrate in striatopallidal and striatonigral neurons specific synaptic adaptations and plasticity such as structural changes associated to different conditions like induction of addictive behavior or motor deficits in PD. It is first worth to note that these mouse lines allowed to identify subtle morphological differences between D_1- and D_2-GFP neurons with higher total dendritic length and higher number of primary dendrites in striatonigral than in striatopallidal neurons (Gertler et al., 2008).

Lee et al. (2006) have demonstrated an increase in spine density both in striatonigral and striatopallidal neurons after chronic methylphenidate and cocaine treatments. However, this increase persisted in striatonigral neurons after 1 month withdrawal whereas it rapidly disappeared in striatopallidal neurons similarly to the increase in ΔFosB expression (Lee et al., 2006), suggesting that the increase of dopaminergic tonus is responsible for the increase of spine density in both MSN sub-populations but with highly different long-term consequences.

On the other hand, Day et al. (2006) demonstrated that 6-OHDA-induced dopamine depletion provoked a selective loss of glutamatergic synapses in striatopallidal neurons, without any spine density modifications in striatonigral neurons, by a mechanism resulting from the dysregulation of the l-type Ca^{2+} channel Cav1.3 in the spine. Such dendritic spine density modification should alter striatopallidal MSN activity and lead to a failure in the correct control of the pallido-subthalamic circuit that could explain motor dysfunction observed in PD. This decrease could be viewed as a homeostatic plasticity to adapt to the decrease in D_2R activation on striatopallidal neurons. This homeostatic hypothesis is also validated by the coincidence in these MSNs of an increased intrinsic excitability and a large decrease in their excitatory synaptic inputs resulting from the loss in dendritic spines in dopamine depletion condition (Azdad et al., 2009a).

Allowing to better understand the homeostatic control of spine density in striatopallidal neurons, Tian et al. (2010) demonstrated that sustained depolarization of co-culture of cerebral cortex and D_2-EGFP striatum induced about 50% loss of dendritic spines and glutamatergic synapses specifically in striatopallidal neurons as seen in the PD animal model (Day et al., 2006). This loss was dependent on Cav1.2, but not on Cav1.3 as in PD model (Day et al., 2006), on activation of calcineurin and up-regulation of one of its target Mef2 which is highly expressed in striatopallidal neurons. This increase induces an up-regulation of two genes, *Nur77* and *Arc*, known to be involved in down-regulation of glutamatergic synapses and inhibition of synapse formation and dendritic spine differentiation (Steward and Worley, 2001; Shalizi et al., 2006; Shepherd et al., 2006). These results therefore identified new mechanisms of activity-dependent synaptic scaling specifically in striatopallidal MSN.

SPECIFIC STRIATOPALLIDAL AND STRIATONIGRAL NEURONS TRANSCRIPTIONAL PROFILES

Genome-wide transcriptional analysis has also recently emerged as a powerful tool to investigate the differential gene expression and gene function of defined cell types.

Recent studies have identified new molecular targets specific to either striatopallidal or striatonigral neurons (Lobo et al., 2006; Heiman et al., 2008). The molecular profiling is an important key to unravel the molecular differences between the two MSN populations in order to better understand their differential functions in the basal ganglia system. By FACS-array profiling of D_1-EGFP and D_2-EGFP neurons, Lobo et al. (2006) identified a subset of new striatopallidal and striatonigral neuron-enriched genes. They have demonstrated the important role of *Ebf1* selectively enriched in striatonigral neurons as regulator of the striatonigral neuron differentiation in the striatal matrix compartment (Lobo et al., 2008). Moreover, they identified a new striatal-specific constitutive G protein-coupled receptor (GPCR), GPR6, which is selectively expressed in striatopallidal neurons. Analysis of GPR6 KO mice demonstrated that this sphingosine-1-phosphate receptor is a critical factor in the striatal production of cAMP. GPR6 KO mice exhibited deficits in instrumental conditioning by which animal learn to obtain a reward by performing a simple task, whereas the locomotor behavior and the motor learning in a rotarod task were normal (Lobo et al., 2007). Therefore, GPR6 seems to be an important and new actor in molecular mechanisms underlying instrumental conditioning and points out the role of striatopallidal neurons in mediating this behavior.

Since gene profiling is therefore a powerful methodology to investigate the differences at the molecular level in striatopallidal and striatonigral neurons and to identify key genes or molecular pathways involved in the basal ganglia physiology, it should also provide new mechanistic data in models of pathological conditions of the basal ganglia circuit as in addiction or PD. Heiman et al. (2008) developed a TRAP (translating ribosome affinity purification) approach consisting in the generation of BAC transgenic mice expressing the EGFP-tagged ribosomal protein L10a specifically in striatopallidal and striatonigral neurons via the *Drd2* and *Drd1a* gene promoters, respectively. After an affinity purification of the EGFP-L10a protein with an antibody against EGFP, the enriched ribosome and the associated mRNA were used to determine the translational profiles of both MSN populations (Heiman et al., 2008). This led to the confirmation of the Lobo's study and the identification of many new genes enriched in either striatopalli-

dal or striatonigral neurons. This subset of genes has been also validated in adult mice by our group using FACS-array profiling of EGFP striatopallidal neurons targeted with the *Adora2a*-Cre mice (Durieux et al., 2009) and striatonigral neurons simultaneously identified by retrograde labeling (Ena et al., unpublished data).

Heiman et al. (2008) approach was also used to track for differential modifications in gene expression profiles in response to pharmacological perturbations. For instance, besides the identification of various genes whose expression has been previously reported to be affected, an up-regulation of the GABA signaling pathway was specifically demonstrated in striatonigral neurons upon chronic cocaine treatment (Heiman et al., 2008). Accordingly, electrophysiological recordings showed specific increase in mIPSC in striatonigral neurons (Heiman et al., 2008). Increased expression in $GABA_A$ receptor in striatonigral neurons could be part of molecular adaptive changes to the increased excitatory drive on these neurons and, hence, could be associated with the modification in sensitivity observed in chronic cocaine addicts. In contrast to this hyperdopaminergic situation upon psychostimulant treatment, Meurers et al. (2009) have recently shown that the expression of multiple components and targets of the cAMP/DARPP-32 signaling pathway are affected in mouse model of PD (6-OHDA mouse model) and after chronic L-DOPA treatment leading to L-DOPA-induced dyskinesia. By using an experimental approach based on a microarray analysis of laser-dissected striatopallidal and striatonigral neurons, these authors identified several gene categories (ion channels, receptors, signaling molecules) affected by the dopamine depletion. These genes were either altered exclusively in one population or in an opposite manner in the striatopallidal and striatonigral neurons. Moreover, Meurers and colleagues highlighted a cell-specific regulation of gene expression in response to chronic L-DOPA treatment, which could give new insight about the mechanism involved in the development of this condition.

EVALUATION OF THE DIFFERENTIAL ROLES OF STRIATOPALLIDAL AND STRIATONIGRAL NEURONS IN MOTOR AND REWARD BEHAVIORS BY CELL-TYPE SPECIFIC ACTIVATION OR ABLATION

According to the classical model of the basal ganglia, the two neuronal MSN populations exert opposite control on motor behavior. Activation of the "direct" pathway would lead to the facilitation of movements whereas the activation of the "indirect" pathway should inhibit the movement. However, the hypothesis was not experimentally validated because of the lack of tools allowing the specific study of these two morphological and intermixed neuronal populations *in vivo*. In the last 2 years, several groups have developed such tools allowing the analysis of functional differences between striatopallidal and striatonigral neurons in motor and reward behaviors. Durieux et al. (2009) have generated a conditional striatopallidal neuron ablation model based on an *Adora2a*-Cre BAC transgenic mouse. The specific ablation was obtained by stereotaxic injection of diphtheria toxin (DT) in the *Adora2a*-Cre mouse crossed with an inducible diphtheria toxin receptor (iDTR) mouse line (Buch et al., 2005). The bilateral injection of DT in the striatum induces a spontaneous hyperlocomotion compared to the control mice, definitively demonstrating the inhibitory role of striatopallidal neurons in the motor activity. Kravitz et al. (2010) recently confirmed this result by using optogenetic control of the two MSN populations. In this approach, adenovirus-associated viruses (AAV1) containing the channel rhodopsin 2 (Chr2) fused to enhanced yellow fluorescent protein (YFP) were injected into the dorsal striatum of BAC transgenic mice expressing the Cre recombinase in striatopallidal or striatonigral neurons (Kravitz et al., 2010; D_2-Cre and D_1-Cre respectively Gong et al., 2007). In D_2-Chr2 mice, illumination of the dorsal striatum induced an activation of striatopallidal neurons leading to a decrease in locomotor initiation and an increase of freezing. In contrast, the activation of striatonigral neurons in D_1-Chr2 mice led to the opposite phenotype, with an increase in locomotor initiation and decrease in freezing (Kravitz et al., 2010). Moreover, bilateral illumination of dopamine-depleted striatum in D_1-Chr2 mice completely restored a normal motor behavior by eliminating bradykinesia, increasing the locomotor initiation, and decreasing freezing; firmly demonstrating the role of the activation of the direct pathway for improvement of motor deficits in PD (Kravitz et al., 2010).

The respective roles of striatopallidal and striatonigral neurons in the control of motivational and reward behavior are so far also poorly understood. However, thanks to the development of conditional tools, several recent studies have successfully addressed this issue. Durieux et al. (2009) have demonstrated that the conditional and specific ablation of striatopallidal neurons in the NAc induced an increase in drug reinforcement as demonstrated by an increased amphetamine conditioned place preference (CPP), strongly suggesting the role of striatopallidal neurons in inhibition of drug reinforcement.

Ferguson et al. (2010) have more recently developed a new system in rat based on the use of viral vector with Enkephalin (*Penk*) or Dynorphyn (*Pdyn*) gene promoters allowing the expression of the new engineered GPCR hMD4 specifically in striatopallidal or striatonigral neurons, respectively. The stimulation of hMD4, a $G_{i/o}$ coupled GPCR, by a specific ligand CNO induces the activation of Kir3 resulting in a membrane hyperpolarization and, hence, a transient neuronal silencing. In this model, administration of CNO in *Penk*-hMD4 or *Pdyn*-hMD4 rats resulted in an increase and a decrease of amphetamine-induced sensitization, respectively (Ferguson et al., 2010). Lobo et al. (2010) have also addressed this question by using optogenetic control of striatopallidal and striatonigral neurons but also by generating conditional loss of TrkB in striatopallidal and striatonigral neurons using D_2-Cre ad D_1-Cre transgenic mice. They demonstrated that the activation of the indirect pathway in a CPP paradigm led to a decrease of the rewarding effect of cocaine whereas the activation of the direct pathway resulted in the opposite phenotype (Lobo et al., 2010). Finally, Hikida et al. (2010) have also targeted striatonigral and striatopallidal neurons by using viral vectors and *Tac1* or *Penk* gene promoters allowing the expression of tTA in transgenic mice containing the TRE–GFP–Tetanus Toxin transgene leading to the inhibition of neurotransmission in targeted neurons. Blockade of the direct pathway showed a decrease in cocaine-induced CPP whereas, in contrast to the studies reported above, no difference in CCP was found when the indirect pathway was inhibited (Hikida et al., 2010). These differences could be explained by the lower proportion (60–70%) of neurons targeted by the two approaches. Altogether, by using different approaches, all based on conditional expression

in MSN populations, these studies demonstrated that similar to their functions in motor control, striatopallidal, and striatonigral neurons display antagonistic roles in the control of reward behavior.

Optogenetic tools have also recently been used to investigate the involvement of NAc cholinergic interneurons in drug reinforcement. By conditional expression of ChR2 or the chloride pump halorhodopsin (eNpHR3.0) in choline acetyltransferase (ChAT)-positive neurons, Witten and colleagues have demonstrated that the activation of cholinergic neurons by stimulation of ChR2 induced the decrease of MSN firing whereas their inhibition by stimulation of eNPHR3.0 induced an increase in MSNs firing in NAc (Witten et al., 2010). The inhibition of ChAT interneurons during cocaine exposure in the conditioning phase of a CPP paradigm induced a significant decrease in cocaine-induced CPP, demonstrating that striatal cholinergic interneurons control NAc MSN activity and consequently regulate cocaine-reward properties (Witten et al., 2010).

EVALUATION OF THE DIFFERENTIAL ROLES OF STRIATOPALLIDAL AND STRIATONIGRAL NEURONS IN MOTOR AND REWARD BEHAVIORS BY CELL-TYPE SPECIFIC GENE OVEREXPRESSION OR INACTIVATION

Thus, the recent development of these powerful techniques allowed to more precisely study the functional roles of striatopallidal and striatonigral neurons. Furthermore, the elucidation of these functional differences was also more and more addressed by using mice with cell-specific knock-out/knock-down of selected genes (i.e., ion channel, receptor) based on BAC-driven Cre recombinase expression or small hairpin RNA (ShRNA).

The first developed conditional striatonigral and striatopallidal models were the NSE-tTa 11A and 11B (tetracycline transactivator) mice which, when crossed with a transgenic line expressing a gene of interest driven by Tet-Op (tetracycline promoter), lead to the induction of this gene expression in the absence of doxycycline (Kelz et al., 1999; Werme et al., 2002). These models were particularly interesting because the gene expression can be induced at adulthood and then avoids developmental effect or gene compensation. Roles of ΔFosB, a transcription factor involved in response to drugs of abuse, have been studied in striatopallidal and striatonigral neurons using this strategy. Overexpression of ΔFosB in striatonigral neurons increased the reward and locomotor response to cocaine and morphine (Kelz et al., 1999; Zachariou et al., 2006) whereas overexpression in striatopallidal neurons did not affect the morphine reward properties (Zachariou et al., 2006), strongly supporting an instrumental role of ΔFosB in striatonigral neurons in reward properties of drugs.

Besides this first conditional model in the basal ganglia system, several groups also developed and used knock-in and BAC transgenic mice to express, under specific striatopallidal or striatonigral neuron promoters, either a protein of interest (Drago et al., 1998; Sano et al., 2003; Gantois et al., 2007), a mutant protein (Heusner and Palmiter, 2005), a shRNA (Novak et al., 2010) or the Cre recombinase used with conditional knock-out floxed mice (Heusner and Palmiter, 2005; Lemberger et al., 2007; Durieux et al., 2009).

The study of NMDA receptor roles in motor and reward functions in the striatum and more specifically in striatonigral neurons is a good example to demonstrate the usefulness of these different approaches. NMDA receptors are involved in the corticostriatal excitatory glutamatergic transmission on MSNs and play a central role in synaptic plasticity at these synapses (Calabresi et al., 1992). By using *Drd1a*- and *Drd2*-EGFP, Shen et al. (2008b) recently demonstrated the role of NMDA receptor in inducing long-term potentiation (LTP) either in striatopallidal or striatonigral neurons and its uselessness in the induction of long-term depression (LTD) in both populations. This could fit with several pharmacological studies showing the implication of striatal NMDA receptors in instrumental learning (Yin et al., 2005). By using a striatal-specific Cre mice (RGS9-Cre) and NMDAR1 (NR1) floxed mice, Dang et al. (2006) have demonstrated that deletion of NR1 subunit in the entire striatum led to a deficit in motor learning in a rotarod task associated with abolition of LTP in the dorsal striatum and LTD in the NAc. Agatsuma et al. (2010), showed more recently the involvement of striatal NR1 subunit in cocaine cue reactivity in a CPP paradigm. However, this model did not allow studying specific involvement of this receptor in the two MSN populations. To more specifically focus on striatonigral neurons, Heusner and Palmiter (2005) developed a knock-in model targeting expression of a NR1 subunit mutant driven by the D_1 receptor promoter (Heusner and Palmiter, 2005) and, more recently, Beutler et al. (2011) crossed knock-in *Drd1a*-Cre mice with NR1 floxed mice. They demonstrated by these two independent approaches that altering NMDA signaling by either NR1 subunit deletion or NR1 mutant expression did not affect basal locomotor activity or acute locomotor response to psychostimulant (Heusner and Palmiter, 2005) but abolished the establishment of cocaine and amphetamine sensitization. Moreover, mice deleted in active NMDA receptors in striatonigral neurons are less sensitive to the rewarding effect of cocaine and amphetamine tested in a CPP paradigm compared to the wild-type littermates (Heusner and Palmiter, 2005; Beutler et al., 2011). Moreover, re-expression of NR1 only in striatonigral neurons by injection of AAV vector in the NAc of *Drd1a*-Cre mice restored amphetamine sensitization, therefore showing that the expression of NR1 in D_1-expressing cells is sufficient to the development of AMPH sensitization (Beutler et al., 2011). In contrast, they also demonstrated that balanced loss of NMDA receptors in both MSN subtypes by using GPR88-Cre mice is permissive for the development of the sensitization. Therefore, removing the NMDA receptors and so compromising the glutamatergic activation in striatonigral neurons leads to an imbalance activity between the two MSN classes that probably promotes the inhibitory role of striatopallidal neurons on amphetamine sensitization. This indicates that an antagonistic balanced activity of striatopallidal and striatonigral neurons is also at works during the establishment of drug sensitization.

Besides ionotropic glutamate receptors, MSNs also highly express members of the group 1 metabotropic glutamate receptors (mGluR; Tallaksen-Greene et al., 1998). MSNs co-express mGluR1 and mGluR5 known to modulate their synaptic activity. mGluR1 and/or mGluR5 are involved in the potentiation of NMDA current, the suppression of GABAergic and glutamatergic transmissions via the activation of presynaptic CB1 receptors as well as in corticostriatal LTP and LTD (for review Bonsi et al., 2008). These forms of plasticity in striatal MSNs are proposed to be associated with motor learning but also associative learning and memory processes that might contribute to relapse like-behavior. The contribution

of mGluR5 in these processes, either in striatonigral neurons or striatopallidal neurons, was investigated through the development of a mGluR5 knock-down mice (mGluR5$^{KD\text{-}D1}$) expressing a small hairpin RNA targeting the mGluR5 under the control of D_1 receptor promoter (Novak et al., 2010). By using a model of cue-induced reinstatement, Novak et al. (2010) showed that mGluR5 in striatonigral MSNs play a role in the reinstatement of cocaine behavior and is therefore required for specific incentive learning processes.

In addition to the central and interrelated roles of glutamatergic and dopaminergic transmissions, muscarinic acetylcholine receptors (*Chmr*) also tightly regulate the basal ganglia network. Disturbances in cholinergic transmission in this system have been suggested in pathologies as Parkinson's disease, depression, schizophrenia, and drug addiction (Felder et al., 2001; Langmead et al., 2008) and *Chmr4* knock-out mice revealed their role in the modulation of the dopaminergic system activity through pre- and/or post-synaptic mechanisms (Gomeza et al., 1999; Tzavara et al., 2004).

Interestingly, *Chmr4* are specifically co-expressed with dopamine D_1 receptor in striatonigral neurons (Bernard et al., 1992; Ince et al., 1997). The physiological relevance of this specific cellular expression was recently analyzed by using the Cre/LoxP system to generate mice lacking *Chmr4* specifically in D_1 receptor-expressing striatonigral neurons (D_1-M4-KO; Jeon et al., 2010). Phenotypical analyses of these D_1-M4-KO mice revealed increase in their response to psychostimulants with increased hyperlocomotion to acute cocaine and amphetamine treatment and increased amphetamine-induced behavioral sensitization. This was accompanied by alterations in dopaminergic transmission, which could contribute to the hypersensitivity phenotype, both at the pre- and post-synaptic levels with an increased dopamine efflux in NAc and a lack of control of the D_1-mediated signaling cascade in striatonigral neurons (Jeon et al., 2010), showing therefore the role of striatonigral MSN-expressed *Chmr4* in the behavioral response to psychostimulants.

In contrast to striatonigral neurons, only few studies have been designed to examine and understand the roles of specific molecules in striatopallidal neurons in behavior. Nevertheless, the involvement of DARPP-32 and BNDF-Trkb signaling in both striatopallidal and striatonigral neurons has been investigated by conditional cell-specific deletion using *Drd2*-Cre and *Drd1a*-Cre mouse lines (Bateup et al., 2010; Lobo et al., 2010). Bateup and colleagues first demonstrated that DARPP-32 is essential for the induction for corticostriatal LTP both in striatopallidal and striatonigral neurons. Further, they showed that conditional deletion of DARPP-32 induces opposite behavioral alterations. Thus, the loss of DARPP-32 in striatopallidal neurons leads to an increased activity whereas this loss in striatonigral neurons results in decreased activity in basal and cocaine-induced locomotor activity. Cell-specific alterations of this signaling cascade highlighted that striatopallidal and striatonigral neurons exert an antagonistic control in mediating locomotor activity and behavioral effect of psychostimulants; striatonigral and striatopallidal neurons facilitating and inhibiting locomotion and rewarding effect of psychostimulants, respectively. Interestingly, only the loss of DARPP-32 in striatonigral neurons fully abolished L-DOPA-induced dyskinesia whilst its deletion in striatopallidal was without effect suggesting the important role of striatonigral DARPP-32 in the L-DOPA-induced dyskinesia. In addition and in the same line, Bateup et al. (2010) demonstrated that DARPP-32 deletion in striatopallidal neurons abolished the haloperidol-induced catalepsy.

BDNF-Trkb signaling has been implicated in the rewarding response to psychostimulants (Grimm et al., 2003; Graham et al., 2007, 2009; Schoenbaum et al., 2007) but the role of the striatopallidal and striatonigral neurons in this mechanism remains unclear. TrkB is expressed by both MSN subtypes but Trkb is significantly enriched in striatopallidal neurons (Lobo et al., 2010). Alteration BDNF-Trkb signaling induced molecular changes as showed by the surprising increase in c-fos expression in striatopallidal neurons and decrease in striatonigral neurons upon acute cocaine treatment while previous studies have demonstrated the selective increase of c-fos expression in striatonigral neurons (Bertran-Gonzalez et al., 2008). This alteration in c-fos expression is associated with a modification in MSN excitability. Striatopallidal neurons displayed a dramatic increase in neuronal firing in basal condition whereas striatonigral neurons displayed this increase only after cocaine treatment, probably due to K^+ channel down-regulation. The increase in striatopallidal activity leads to the desensitization of the rewarding effect of cocaine whereas increase excitability of striatonigral neurons promotes the effect of cocaine (Lobo et al., 2010). Cell-specific deletion of Trkb confirmed the antagonistic role of striatopallidal and striatonigral neurons in the locomotor response to cocaine treatment, which is consistent with current models of basal ganglia circuit (Lobo et al., 2010).

As indicated above, Lobo et al. (2007) studied the new striatopallidal neuron-specific gene *Gpr6* by using full knock-out mice highlighting its involvement in the control of the cAMP signaling cascade in these neurons and its role in instrumental learning. Involvement of adenosine A_{2A} receptor, a well-known striatopallidal neuron-specific gene (Schiffmann and Vanderhaeghen, 1993; Schiffmann et al., 2007), has been extensively studied by using pharmacological tools and knock-out mice leading to apparent discrepant results. Contrary to the antagonist A_{2A}-D_2 interaction model in the striatum (Ferre et al., 1997), based on various data including electrophysiological studies showing antagonistic A_{2A}-D_2 control of D_2-GFP MSN membrane potential oscillations through A_{2A}-D_2 receptors heteromerization (Azdad et al., 2009b) and pharmacological studies (Filip et al., 2006), global genetic deletion (Chen et al., 2000, 2003; Soria et al., 2006) or more specific deletion in the forebrain (using *CamKIIα*-Cre mouse line and floxed A_{2A} receptor mice; Bastia et al., 2005) led to an attenuation rather than a increase of the locomotor effect induced by psychostimulants. Reconciling these different results, deletion of A_{2A} receptor specifically at the post-synaptic level in striatal neurons by using *Dlx5-6*-Cre mice showed that, in contrast to forebrain deletion, acute administration of cocaine resulted in an enhanced cocaine-induced locomotor activity (Shen et al., 2008a), that could be explained by the antagonistic interaction between A_{2A}R and D_2R in striatopallidal neurons. These results showing opposite effects of pre- and post-synaptic A_{2A}R nicely illustrate the importance of having adequate tools to inactivate genes in specific neuronal populations to decipher their functions. In addition, using devaluation omission behavioral assay for habit formation, Yu et al. (2009) demonstrated that A_{2A}R deletion in striatopallidal

Table 1 | Behavioral phenotype in striatopallidal neuron-specific transgenic mouse models.

Specific neuronal inhibition or ablation model	Specific neuronal activation model (optogenetic tools)
Increased basal locomotor activity (Durieux et al., 2009) Increased rewarding effect of amphetamine (Durieux et al., 2009) Increased amphetamine sensitization (Ferguson et al 2010)	Decreased basal locomotor activity and increased freezing (Kravitz et al., 2010) Decreased rewarding effect of cocaine (Lobo et al., 2010)
SPECIFIC GENE TARGETING	
Increased basal locomotor activity in DARPP-32 conditional knock-out mice (Bateup et al., 2010)	Habit formation alteration in striatopallidal neuron-A_{2A}R knock-out (Yu et al., 2009)
Increased acute cocaine-induced hyperlocomotion in DARPP-32 conditional knock-out mice (Bateup et al., 2010)	Instrumental conditioning affected by GPR6 deletion (Lobo et al., 2007)
Decreased rewarding effect of cocaine in TrkB conditional knock-out mice (Lobo et al., 2010)	Disruption of haloperidol-induced catalepsy (Bateup et al., 2010)

Table 2 | Behavioral phenotype in striatonigral neuron-specific transgenic mouse models.

Specific neuronal inhibition model (optogenetic tools)	Specific neuronal activation model (optogenetic tools)
Decreased amphetamine sensitization (Ferguson et al., 2010) Increased rewarding effect of cocaine (Lobo et al., 2010)	Increased basal locomotor activity and decreased freezing (Kravitz et al., 2010)
SPECIFIC GENE TARGETING	
Decreased basal locomotor activity, acute cocaine-induced hyperlocomotion and l-DOPA-induced dyskinesia in DARPP-32 conditional knock-out mice (Bateup et al., 2010)	Alteration of cue-induced reinstatement in striatonigral neuron mGluR5 knock-down mice (Novak et al., 2010)
Increased locomotor response and rewarding effect of cocaine and morphine by ΔFosB overexpression (Zachariou et al.,2006)	Increased cocaine and amphetamine acute hyperlocomotion and behavioral sensitization in D1-M4-KO (Jeon et al., 2010)
Increased rewarding effect of cocaine in TrkB conditional knock-out mice (Lobo et al.,2010)	Abolished sensitization and decreased rewarding effect of psychostimulant in striatonigral neuron NR1 subunit-lacking mice (Beutler et al., 2011)

neurons led to a selective deficit in instrumental learning and hence revealed the importance of A_{2A}R-mediated signaling cascade in striatopallidal neurons for habit formation.

CONCLUSION

Data summarized in this review extraordinarily extend our understanding of signaling pathways and mechanisms that are selectively activated in striatopallidal and striatonigral neurons in response to different stimuli, demonstrating the differential implication of these neuron sub-populations in the control of motor and drug-induced behaviors (see **Tables 1 and 2**). This rapid extension was render possible by the critical development of new technologies in the last decade allowing to specifically identify and study these two key neuronal populations in the striatum.

First, specific identification of striatopallidal and striatonigral neurons has given the opportunity to investigate more precisely molecular pathways involved in different conditions, i.e., in psychostimulant responses. Furthermore, genome wide analysis of striatal MSNs provided a huge number of new genes and molecular pathways that are specifically present in striatopallidal and striatonigral neurons. The functional relevance of these genes and pathways represent an important challenge that will be analyzed in the future. Finally, the development of cell-specific models using Cre lines and conditional floxed mice is profoundly changing the way we analyze basal ganglia functions and allows to much better investigate striatopallidal and striatonigral neuron properties and to dissect molecular mechanisms underlying their differential functions in the basal ganglia circuit.

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Cell signaling underlying epileptic behavior

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Epilepsy is a complex disease, characterized by the repeated occurrence of bursts of electrical activity (seizures) in specific brain areas. The behavioral outcome of seizure events strongly depends on the brain regions that are affected by overactivity. Here we review the intracellular signaling pathways involved in the generation of seizures in epileptogenic areas. Pathways activated by modulatory neurotransmitters (dopamine, norepinephrine, and serotonin), involving the activation of extracellular-regulated kinases and the induction of immediate early genes (IEGs) will be first discussed in relation to the occurrence of acute seizure events. Activation of IEGs has been proposed to lead to long-term molecular and behavioral responses induced by acute seizures. We also review deleterious consequences of seizure activity, focusing on the contribution of apoptosis-associated signaling pathways to the progression of the disease. A deep understanding of signaling pathways involved in both acute- and long-term responses to seizures continues to be crucial to unravel the origins of epileptic behaviors and ultimately identify novel therapeutic targets for the cure of epilepsy.

Keywords: Fos, ERK, activity-dependent transcription, seizure, apoptosis, hippocampus

BEHAVIORAL MANIFESTATIONS OF SEIZURES AND EPILEPSY

Epilepsy is one of the most common neurological disorders, characterized by the repeated occurrence of sudden and transitory episodes of motor, sensory, autonomic, and psychic origin, known as seizures. Seizures typically arise in restricted regions of the brain and may remain confined to these areas ("focal" or "partial" seizures) or spread to the whole cerebral hemispheres ("generalized" seizures). The behavioral outcome of seizure events depends on the brain regions that are affected by overactivity. This is clearly exemplified by the behavioral manifestations of temporal lobe epilepsy (TLE), which represents one of the most common forms of human epilepsy. In TLE, focal seizures arise in a restricted part of the limbic system (temporal lobe: hippocampus, parahippocampal gyrus, and amygdala). If paroxysmal activity remains confined to the area of onset ("simple partial" seizure), the seizure may be characterized by auditory, gustatory, olfactory, visual, or somatosensory hallucinations (auras), accompanied by psychic sensations such as euphoria, fear, and anger. The seizure may also spread to a larger portion of the temporal lobe ("complex partial" seizure), resulting in impaired consciousness, motionless staring, motor automatisms of the hands or mouth, altered speech, and other unusual behaviors. Finally, partial seizures arising in the temporal lobe may subsequently spread to the whole brain ("secondarily generalized tonic–clonic" seizures or "grand mal" seizures). These seizures begin with symptoms of a partial seizure followed by whole-body convulsions (Berg et al., 2010).

Temporal lobe epilepsy can be reproduced in laboratory animals (typically rodents) by the systemic or intracerebral administration of powerful convulsant agents such as glutamatergic (kainic acid) or cholinergic (pilocarpine) agonists (Pitkänen et al., 2005). According to the classical scale originally proposed by Racine (1972), experimental seizures evoked in the temporal lobe in rodents can be scored on the basis of specific behaviors that correspond to different brain regions impacted by seizure activity. Stages from 1 to 3 are characterized by behavioral signs of focal seizure activity restricted to the temporal lobe (hippocampal formation, entorhinal cortex, and amygdala): immobility, rigid posture, forelimb and/or tail extension, repetitive movements, and head bobbing. When activity generalizes and spreads to cerebral hemispheres, isolated limbic motor seizures (forelimb clonus with rearing and falling; stage 4) or continuous convulsive activity (*status epilepticus*, stages 5–6: continuous rearing and falling, whole-body tonic–clonic convulsions) occur. Electroencephalographic (EEG) and metabolic mapping studies confirmed these behavioral observations. EEG analysis of experimentally induced limbic seizures showed that epileptic activity sequentially appears in forebrain areas, starting from the hippocampus and then spreading to the amygdala, and cerebral cortex (Lothman et al., 1981; Turski et al., 1989). Consistent with these results, glucose utilization studies in rodents at different times after limbic seizure induction revealed a progressive increase in several forebrain areas, starting in the hippocampus, amygdala, and other limbic areas and then diffusing to cerebral cortical areas (Lothman and Collins, 1981; Clifford et al., 1987).

In the past 25 years, a large series of studies addressed the crucial issue of how neurons transduce pathological electrical activity from their membrane to the nucleus, resulting in both short- and long-term rearrangements that modify neuronal connectivity in the epileptic brain and, ultimately, brain function, and behavior. Many of these mechanisms are now well characterized, whereas others still remain to be clearly understood. In the following sections, we will describe some of the major signaling pathways involved in short- and long-term cellular responses to seizure activity that are thought to underlie acute and chronic behaviors in the epileptic brain.

THE ACUTE CELLULAR RESPONSE TO SEIZURES: ACTIVATION OF IMMEDIATE EARLY GENES

The first demonstration that pathological overactivity can modify gene expression in the brain came from the pioneering studies by J. I. Morgan and T. Curran, who showed that metrazole-induced seizures markedly induce c-*fos* mRNA expression in several areas of the rodent brain (Morgan et al., 1987). These authors first introduced the concept that neurons largely use the rapid activation of "immediate early genes" (IEGs; usually transcription factors, such as Fos and Jun) to couple acute and long-term responses to physiological as well as pathological stimuli (Morgan and Curran, 1989, 1991a). Induction of activity-regulated transcription factors is a general phenomenon occurring in neurons after acute seizures (Morgan and Curran, 1991b; Herrera and Robertson, 1996; Hughes et al., 1999). However, c-*fos* certainly remains the prototypical and well characterized activity-dependent transcription factor, and its induction is widely considered a suitable marker of neuronal activity. As originally demonstrated using fos-lacZ transgenic mice, seizures induce c-*fos* mRNA transcription in defined neuronal populations at different times (Smeyne et al., 1992). These observations have been confirmed by several studies using c-*fos* mRNA *in situ* hybridization or c-Fos immunostaining on rodent brain sections as a way to perform activity mapping studies after seizures. A precise correlation exists between the pattern of c-*fos* induction and the progression of seizures from focal to generalized. Focal epileptic activity stimulates c-*fos* mRNA and c-Fos protein induction only in a few limbic areas, typically initiating in granule cell layer of the dentate gyrus and then spreading to CA3 and CA1 pyramidal layers. Then, when activity generalizes and limbic motor seizures and *status epilepticus* occur, a widespread c-*fos* mRNA and c-Fos protein expression is detected throughout the whole cerebral cortex and several other brain areas (Barone et al., 1993; Willoughby et al., 1997; Bozzi et al., 2000; Tripathi et al., 2008). More recent findings suggest that the increased level of phosphorylated ERK (pERK) could be one of the earliest immunohistochemical indicators of neurons that are activated at the time of a spontaneous seizure (Houser et al., 2008). In spontaneously epileptic animals, a marked increase in pERK labeling occurred at the time of spontaneous seizures and was evident in large populations of neurons at very short intervals (as early as 2 min) after detection of a behavioral seizure.

The intracellular signaling cascades involved in IEGs activation in both physiological and pathological conditions have been extensively investigated in neurons. So far, the pathways involved in c-*fos* induction remain the best characterized and can be briefly summarized as a prototypical example of activity-dependent neuronal gene transcription. Neuronal depolarization leads to increased intracellular levels of the second messengers cAMP (typically, following neurotransmitter/neuromodulator binding to G-protein coupled receptors) and Ca^{2+} (e.g., due to ion channel opening following glutamate binding to glutamate receptors). Both these two second messengers activate intracellular kinases [protein kinase A and extracellular-regulated kinases (ERK)] whose activity converges on the phosphorylation of the transcription factor CREB (cAMP response element binding protein, constitutively present in the nucleus). In turn, CREB phosphorylation activates c-*fos* mRNA transcription. c-*fos* mRNA is then translated into the c-Fos protein, that acts as a transcription factor for a wide variety of neuron-specific genes (reviewed in West et al., 2002; Flavell and Greenberg, 2008). This mechanism is rapid, and allows neurons to fast couple depolarizing stimuli to a wide variety of intracellular long-lasting responses, including the induction of genes involved in synaptic plasticity and cell death (see below).

Intracellular cascades activated by seizures are largely overlapping those involved in synaptic plasticity, and more specifically in long-term memory (that requires IEGs induction and new protein synthesis). These cascades have been widely studied in the hippocampus, that is crucially involved in learning and memory, but is also one of the most epileptogenic brain areas. From these studies it emerges that hippocampal neurons use the same signaling pathways to respond to both physiological and pathological stimuli. However, these pathways are harmful only when activated by seizures and not physiological neuronal activity. Different types of activity pattern might elicit physiological or pathological responses of hippocampal neurons. For example, only epileptogenic stimuli (such as kainic acid and pilocarpine), but not other types of electrical stimulation, are able to rapidly (3 h) induce dendritic accumulation of BDNF mRNA and protein in hippocampal neurons (Tongiorgi et al., 2004), a change that is thought to contribute to the pro-epileptogenic action of BDNF (Simonato et al., 2006).

SIGNALING PATHWAYS ACUTELY INDUCED BY SEIZURES: THE ROLE OF NEUROMODULATORS

Seizures have been traditionally characterized as an imbalance between excitatory (glutamatergic) and inhibitory (GABAergic) transmission. The role of glutamate, GABA, and their respective signaling pathways in seizures and epilepsy has been extensively addressed in the literature (see for example McNamara et al., 2006; Ben-Ari et al., 2007) and will not be further reviewed here. Instead, we focus on the role that neuromodulatory systems have been shown to regulate seizure activity. The activities of dopamine, noradrenaline, and serotonin systems in modulating seizure threshold have been widely investigated, and all these neuromodulators have been shown to positively or negatively regulate the generation of seizures, depending on the receptors involved. However, the nature of the coupling of their receptors to intracellular signal transduction and, in particular, the induction of IEGs is still being investigated. Here, we describe the current state of knowledge of cell signaling pathways activated by neuromodulators and involved in seizure onset and propagation.

CATECHOLAMINES: DOPAMINE AND NORADRENALINE

The role of catecholamines (dopamine and noradrenaline) in the control of seizure onset and propagation has been widely addressed in experimental, clinical, and therapeutic studies (Starr, 1993, 1996; Weinshenker and Szot, 2002; Giorgi et al., 2004). However, the topic has received very little attention regarding the underlying signaling pathways activated by seizures. No data are available on the signaling cascades downstream of noradrenaline receptors during seizures, with the exception of a recent study addressing the role of noradrenergic neurons of the locus coeruleus (LC) in limbic seizure activity. LC neurons densely innervate limbic areas of the brain, greatly contributing to control seizure activity. LC neurons play an important role in determining IEG expression following seizures. Indeed, LC lesion markedly reduces seizure-induced Fos expression

in the hippocampus, indicating that noradrenergic inputs to the limbic system positively control IEG transcription during seizures (Giorgi et al., 2008).

More studies instead investigated the signaling pathways downstream of dopamine receptors that might contribute to limbic seizure onset and spread. Dopamine acts through two different types of G-protein coupled receptors, named D1-like and D2-like receptors. Activation of D1-like (D1 and D5) receptors results in reduction of seizure threshold and increased seizure severity (DeNinno et al., 1991). Administration of a sub-threshold dose of pilocarpine in the presence of D1 receptor agonists has been shown to induce seizures (Starr and Starr, 1993). Conversely, the effect of D2-like (including D2, D3, and D4) receptors on seizure modulation is predominantly inhibitory. Administration of D2-like receptor agonists results in lower seizure activity, whereas blockade or genetic inactivation of these receptors has pro-convulsant effects (Starr, 1993, 1996; Bozzi et al., 2000; An et al., 2004; Bozzi and Borrelli, 2002, 2006).

The canonical pathway of dopamine receptor activity in neurons involves modulation of adenylate cyclase (AC) to regulate cAMP production and subsequent activation of protein kinase A (PKA). D1-like receptors are coupled to Gs/olf proteins and stimulate AC, leading to increased levels of the second messenger cAMP. Conversely, D2-like receptors are coupled to Gi/o proteins and inhibit AC, decreasing cAMP production (Callier et al., 2003). Pharmacological and targeted gene knockout studies provided some insight into the profile of these pathways explicitly in seizures. It appears that specific receptor coupling to AC may be critical in seizure induction by D1-like receptors (O'Sullivan et al., 2005). Stimulation of phospholipase C (PLC) signaling, however, does not appear to have any effect on seizure threshold (Clifford et al., 1999) and is associated with more subtle behaviors. Dopamine and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) has been identified as one of the critical downstream targets of dopamine and PKA. PKA-catalyzed phosphorylation activates DARPP-32, converting it into an inhibitor of protein phosphatase-1 (PP-1). Specifically, casein kinase 1 (CK1) promotes the activation of DARPP-32 by reducing calcineurin-dependent dephosphorylation at the PKA site. Phosphorylated DARPP-32, by inhibiting PP-1, acts together with other protein kinases (mainly PKA and PKC) to increase the level of phosphorylation of a number of downstream effector proteins, including GABA and glutamate receptors, Ca^{2+} and Na^{+} channels, and the transcription factor CREB (Greengard et al., 1999; Greengard, 2001). DARPP-32 phosphorylation was observed in mice treated with D1-like receptor agonists and this elevation correlated with seizure behavior (O'Sullivan et al., 2008). The crucial role of DARPP-32 in mediating dopaminergic control of seizures was highlighted by the observation that seizure behaviors were absent or greatly reduced in DARPP-32 knockout mice (O'Sullivan et al., 2008).

As outlined previously, D2-like receptor stimulation appears to have an antagonistic effect to D1-like stimulation. Also acting on AC, it can inhibit the activity of cAMP and reduce PKA activation (Missale et al., 1998). Moreover, D2 receptor (D2R) activation has been shown to downregulate DARPP-32 activity (Nishi et al., 1997). Recent evidence, however, suggests a role of an alternative pathway in D2R-modulated seizure behavior. Studies in the striatum suggested that a cAMP-independent pathway involves the formation of a signaling complex composed of D2R, Akt, protein phosphatase-2A (PP2A), and β-arrestin 2. Activation of this pathway following binding of dopamine to D2R results in dephosphorylation of Akt (inactivation), followed by dephosphorylation (activation) of glycogen synthase kinase 3β (GSK-3β; Beaulieu et al., 2005, 2007). Similar activation of GSK-3β was observed in the hippocampus following KA administration in D2R knockout ($D2R^{-/-}$) mice (Tripathi et al., 2010), suggesting that upregulation of GSK-3β activity might contribute to increased susceptibility to seizure-induced cell death observed in these mice. Indeed, activation of GSK-3β has been implicated in neuronal cell death (Kaytor and Orr, 2002). Importantly, GSK-3β upregulation was independent of any change in Akt in $D2R^{-/-}$ mice following seizures (Tripathi et al., 2010), implicating that alternative pathways might contribute to modulate GSK-3β in the hippocampus during epileptic activity. p38MAPK and Wnt pathways have both been implicated as potential alternative pathways in regulating GSK-3β activity (Thornton et al., 2008; Inestrosa and Arenas, 2010), but require further investigation in the context of seizures.

In addition to its direct role on glutamatergic excitation (see above), ERK signaling is also believed to play an integral role in D1- and D2-type receptor signaling. Upregulation of ERK and increased phosphorylation of the ribosomal protein S6 and histone H3 (two downstream targets of ERK) were detected in the granule cell layer of the dentate gyrus, in response to brief seizures evoked by D1 specific agonists; these effects were prevented by genetic inactivation of D1 receptor, or by pharmacological inhibition of ERK (Gangarossa et al., 2011). Importantly, D1 receptor-mediated ERK upregulation disappeared within 60 min and correlated with seizure activity (Gangarossa et al., 2011). This is in contrast to kainic acid or pilocarpine-induced ERK upregulation, which can persist for hours (Kim et al., 1994; Berkeley et al., 2002). Thus, brief or long-lasting ERK activation in selective epileptogenic areas of the brain might contribute to activate signaling pathways in response to different seizure-promoting stimuli, as well as different types of seizure behaviors. Spontaneous chronic seizures following pilocarpine treatment rapidly induce ERK phosphorylation in the dentate gyrus specifically in a subpopulation of cells in the subgranular zone, that were identified as proliferating radial glia-like neural precursors (Li et al., 2010b). The functional significance of ERK activation, and its link with the detrimental or beneficial effects of seizure-induced neurogenesis (Kokaia, 2011), remain to be clarified.

Seizure-induced ERK activation seems to be controlled also by D2R-dependent pathways. Following kainic acid treatment, more rapid and longer-lasting ERK phosphorylation is detected in the hippocampus of $D2R^{-/-}$ mice, as compared to wild-type controls (Yuri Bozzi, unpublished observations). Downstream of ERK, the induction of IEGs is a critical effector step and has been extensively studied in seizure models (see above). D1-type receptor agonists increase the levels of Zif268 and Arc/Arg3.1 (two IEGs involved in transcriptional regulation and synaptic plasticity) in the dentate gyrus, with a time course that parallels that of ERK phosphorylation (Gangarossa et al., 2011).

Studies performed in $D2R^{-/-}$ mice confirm that dopaminergic innervation to the hippocampus plays a crucial role in seizure-induced IEGs induction. Experimentally induced seizures in these mice result in a more prominent upregulation of c-*fos* and c-*jun* mRNAs in the hippocampus, as compared to wild-type controls (Bozzi et al., 2000). As observed following administration of D1

agonists in wild-type mice (Gangarossa et al., 2011), seizure-induced IEGs upregulation in D2R$^{-/-}$ mice was transient in the dentate gyrus (Bozzi et al., 2000), confirming the critical role of this structure in mediating the first steps of dopamine-dependent control of hippocampal activity during seizures.

SEROTONIN

Serotonin (5-hydroxytryptamine, 5-HT) acts through a family of predominantly G-protein coupled receptors to modulate neuronal excitability (Barnes and Sharp, 1999; Hannon and Hoyer, 2008). Activation of 5-HT receptors by administration of 5-HT agonists or reuptake inhibitors can inhibit focal and generalized seizures, while destruction of serotonergic terminals and depletion of brain 5-HT results in reduction of seizure threshold and increased neuronal excitability in experimental models (Bagdy et al., 2007). More recently, increased threshold to kainic acid-induced seizures was observed in mice with genetically increased 5-HT levels (Tripathi et al., 2008).

Despite the well established role of 5-HT in seizure modulation, the receptor subtypes and signaling pathways through which 5-HT exerts its effect are not well characterized. Fourteen mammalian 5-HT receptor subtypes are currently recognized, and these have been classified into seven receptor families on the basis of their structural, functional and, to some extent, pharmacological characteristics (Barnes and Sharp, 1999; Hannon and Hoyer, 2008). Among these receptors, the 5-HT$_{1A}$ and 5-HT$_{2C}$ subtypes, which are expressed in epileptogenic brain areas (mainly, cerebral cortex and/or hippocampus), appear to be the most relevant in epilepsy (Bagdy et al., 2007). For example, increased seizure severity is observed in mice with targeted inactivation of the 5-HT$_{1A}$ or 5-HT$_{2C}$ genes (Tecott et al., 1995; Brennan et al., 1997; Sarnyai et al., 2000). However, it must be noted that pharmacological studies using the 5-HT$_1$ or 5-HT$_2$ specific agonist/antagonists failed to give a clear evidence of the general role played by these receptors in seizure modulation: contrasting results were obtained depending on the experimental seizure models used to test the anti- or pro-convulsant effect of different ligands. A detailed description of these findings is beyond the scope of this review and the reader is referred to more comprehensive previous studies (Bagdy et al., 2007).

Similar to dopamine, most of 5-HT receptors act on the AC/cAMP/PKA pathway. The only exceptions are 5-HT$_2$ receptors (that modulate PLC) and the 5-HT$_{3A}$ receptor (a ligand-gated ion channel; Bockaert et al., 2006; Hannon and Hoyer, 2008). 5-HT receptor signaling modulates IEGs induction in response to excitatory glutamatergic stimuli, such as those resulting from seizure activity. For example, the induction of Arc/Arg3.1 and c-*fos* in cortical neuronal populations following treatment with DOI (a 5-HT$_2$ receptor agonist) is critically dependent on glutamate receptor activation (Pei et al., 2004). As regarding the neuromodulatory role of 5-HT on intracellular signaling pathways acutely induced by seizures, it is interesting to note that activation of the 5-HT$_2$/PLC pathway leads to phosphorylation of DARPP-32 (Svenningsson et al., 2002). The observation that pharmacological and genetic manipulations of 5-HT$_2$ receptors modulate seizure activity (see above; Bagdy et al., 2007) suggests that DARPP-32 may act as a converging point of the neuromodulatory action of both dopamine and 5-HT in seizure-induced signaling cascades.

Figure 1 summarizes the major signaling pathways downstream of dopamine and 5-HT receptors in the early response to seizures.

SEIZURE-INDUCED CELL DEATH: INTRINSIC AND EXTRINSIC PATHWAYS

Studies performed in both experimental models and findings in humans support the notion that seizures can be damaging to the brain. In particular, if seizures are prolonged (*status epilepticus*) or repeatedly evoked they can cause neuronal death, particularly within vulnerable brain

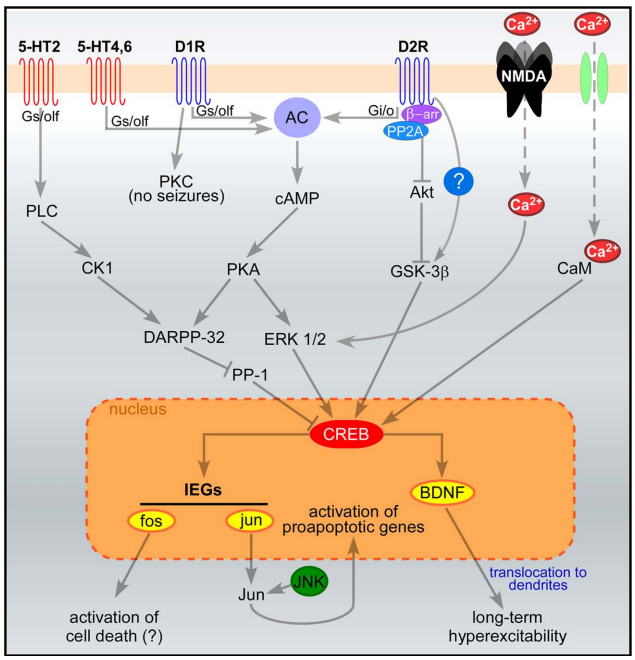

FIGURE 1 | Signaling pathways acutely activated in hippocampal neurons following seizures. Pathways downstream of glutamate, serotonin and dopamine receptors are illustrated. Seizures induce massive influx of Ca^{2+} through NMDA receptors and voltage-gated Ca^{2+} channels (in green), leading to CREB phosphorylation via ERK and calmodulin-dependent signaling, respectively (West et al., 2002). Serotonin and dopamine signaling modulate seizure-induced CREB phosphorylation via the activation of DARPP-32 and ERK1/2. Once phosphorylated, CREB promotes the transcription of activity-dependent genes such as BDNF and the IEGs *fos* and *jun*. The sustained induction of *jun* has been shown to switch on apoptotic cascades, whereas the pro-apoptotic role of *fos* induction has been questioned. Dendritic localization of BDNF mRNA and protein may also contribute to long-term excitability. The proposed scheme is a general (though not complete) summary of the intracellular pathways induced by seizures in the hippocampus. All the reported serotonin and dopamine receptor subtypes are expressed in the hippocampus, together with their signaling proteins (Meador-Woodruff et al., 1991; Perez and Lewis, 1992; Hannon and Hoyer, 2008). However, important differences may occur in different types of hippocampal neurons (e.g., dentate granule cells, pyramidal neurons), due to the different expression levels of these proteins. Abbreviations: AC, adenylate cyclase; CaM, calmodulin; CK1, casein kinase 1; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; D1R and D2R, dopamine receptors (D1 and D2 subtypes); ERK, extracellular-regulated kinase; GSK-3β, glycogen synthase kinase 3β; IEGs, immediate early genes; JNK, Jun-terminal kinase; NMDA, NMDA glutamate receptors; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PP-1, protein phosphatase 1; 5-HT, serotonin receptors. Question marks indicate that some pathways have been proposed but not clearly demonstrated.

regions such as the hippocampus. Cell death is a common pathologic feature of insults to the brain which trigger a chronic epileptic condition (Pitkanen and Sutula, 2002). The main mechanism by which neurons die after seizures is excitotoxicity due to prolonged over-activation of ionotropic glutamate receptors (Choi, 1988; Meldrum, 1991; Fujikawa, 2005). An appreciation of the complex molecular pathways lying downstream of glutamate receptor over-activation has emerged, alongside evidence that glutamate receptor antagonists do not block all cell death after seizures (Bengzon et al., 1997).

Several studies have addressed the role of IEGs in seizure-induced cell death (Herdegen and Leah, 1998). The prolonged activation of the IEGs c-*fos* and c-*jun* after acute seizures was originally proposed as one of the crucial steps that trigger long-term neuronal death (Smeyne et al., 1993; Kasof et al., 1995; Kondo et al., 1997). In physiological conditions, IEGs are essential for normal neuronal responses to excitation in the brain. The protein products of fos/jun contribute to assembly of the AP-1 transcription factor, whose activation regulates the expression of a wide number of "synaptic" genes, including neurotrophic factors (Sheng and Greenberg, 1990). Indeed, loss of c-*fos* in mice markedly reduces activity-dependent structural plasticity (mossy fiber sprouting; Watanabe et al., 1996). This suggests that such a "fos-less phenotype" might be due to altered seizure-induced transcriptional activation of genes involved in synaptic plasticity (Watanabe et al., 1996). However, the role of c-*fos* as a promoting agent of seizure-induced neuronal cell death has been questioned by the observation that conditional mutant mice lacking c-*fos* in the hippocampus display increased susceptibility to seizure-dependent neuronal loss (Zhang et al., 2002). Conversely, the pro-apoptotic activity of c-*jun* activation has been confirmed in several experimental models. Increased levels of both c-*jun* mRNA and Jun phosphorylation are observed in epileptogenic areas after seizures (Huges et al., 1999; Raivich and Behrens, 2006). Phosphorylation of Jun is mediated by the c-Jun N-terminal kinase (JNK) and results in the subsequent activation of Jun transcriptional activity, that triggers apoptotic neuronal cell death after seizures (Herdegen et al., 1998; Behrens et al., 1999; Mielke et al., 1999; Schauwecker, 2000; Spigolon et al., 2010) and other traumatic insults (Yuan and Yankner, 2000). Accordingly, mice lacking the Jun-terminal kinase Jnk3 are protected against seizure-induced apoptosis in the hippocampus (Yang et al., 1997).

Apoptosis-associated signaling pathways are important components of the molecular response to seizures (Engel and Henshall, 2009). Complex intracellular and intercellular cell death-regulatory pathways are increasingly recognized as important contributors to seizure-induced neuronal death. Here, we review the latest findings on apoptosis-associated signaling pathways, in particular the role of Bcl-2 family proteins, as critical components of the molecular response to seizures.

Apoptosis is a form of programmed cell death that is orchestrated by evolutionarily-conserved signaling pathways. Two main pathways have been identified. The intrinsic pathway is activated by various intracellular stressors such as DNA damage, perturbed intracellular organelle function (for example, prolonged endoplasmic reticulum stress), and loss of calcium homeostasis (Orrenius et al., 2003; Xu et al., 2005). Ultimately, this results in mitochondrial outer membrane permeabilization (MOMP) and release of apoptogenic molecules from mitochondria, in particular cytochrome c. Having transited from the inter-membrane space to the cytoplasm, cytochrome c binds the apoptotic protease activating factor 1 (Apaf-1) which recruits caspase-9, a long pro-domain-containing member of the caspase family of cysteinyl aspartate-specific proteases. This forms the so-called apoptosome which processes downstream effector caspases such as caspase-3, culminating in cleavage of various structural and other proteins and, finally, cell death. The extrinsic pathway is activated when cytokines such as Fas ligand bind surface-expressed death receptors of the tumor necrosis factor (TNF) superfamily (Ashkenazi and Dixit, 1998). Upon binding, receptors recruit intracellular adaptor proteins such as Fas-associated death domain protein (Fadd) which can activate caspase-8, followed by effector caspases (Wilson et al., 2009).

Apoptosis-inducing factor (AIF) is a critical mediator of caspase-independent apoptosis. AIF is released from mitochondria whereupon it translocates to the nucleus and mediates large scale DNA fragmentation and nuclear condensation (Hangen et al., 2010). AIF release is triggered by various insults including excitotoxicity and AIF can be blocked in some models by Bcl-2 (Susin et al., 1999).

Bcl-2 FAMILY

Control over MOMP and the downstream caspase-dependent and, possibly, AIF/caspase-independent cell death process, rests with the Bcl-2 family proteins. The family is characterized by the presence of one or more Bcl-2 homology (BH) domains and is divided into anti-apoptotic members, including Bcl-2 and Bcl-XL, and pro-apoptotic members. Within the pro-apoptotic family there are the multi-BH domain proteins, Bax and Bak, and the BH3-only subgroup. BH3-only proteins are the sentinels of cell stress. At least 10 members have been identified which share homology within the short BH3 domain but are otherwise structurally unrelated (Youle and Strasser, 2008). Some members are constitutively expressed in cells, requiring release from chaperones or post-translational modification to become active. Others are not normally present and require transcriptional upregulation. Two non-mutually exclusive mechanisms have been proposed for how BH3-only proteins promote MOMP. First, BH3-only proteins bind to anti-apoptotic members thereby neutralizing them. This displaces and allows activation of the multi-BH domain proteins Bax/Bak to trigger MOMP. A second mechanism is the direct activator model whereby BH3-only proteins directly activate Bax/Bak by binding and promoting their integration into mitochondria (Youle and Strasser, 2008; Chipuk et al., 2010). There is a hierarchy among BH3-only proteins with so-called "weak" members such as Bad having restricted affinity for only few anti-apoptotic Bcl-2 family proteins and unable to directly activate Bax/Bak, whereas potent members of the BH3-only family including Bim, Bid, and probably Puma can avidly bind all anti-apoptotic Bcl-2 family proteins and directly activate Bax/Bak (Youle and Strasser, 2008; Chipuk et al., 2010). Activation of pro-apoptotic Bcl-2 family proteins, in particular Bax and Bid, may also directly cause mitochondrial fragmentation that contributes to cell death (Youle and Karbowski, 2005).

INDUCTION OF APOPTOSIS-ASSOCIATED SIGNALING PATHWAYS AFTER ACUTE SEIZURES

There is substantial evidence that prolonged or repeated seizures activate apoptosis-associated signaling *in vivo* (Engel and Henshall, 2009). Within a few hours of *status epilepticus* there is release of cytochrome *c* and other pro-apoptogenic molecules from

mitochondria and an ensuing caspase cascade, and neuronal death can be interrupted using caspase inhibitors (Henshall et al., 2000a, 2001a,b; Viswanath et al., 2000; Narkilahti et al., 2003a; Li et al., 2006; Manno et al., 2007; Murphy et al., 2007).

Seizures also activate caspase-independent apoptosis pathways in the brain (Cheung et al., 2005; Takano et al., 2005; Engel et al., 2010a; Zhao et al., 2010). Here, calpains are thought to be critical. There are over 60 known calpain substrates and calpain is viewed as a critical coordinator of calcium-dependent signaling pathways underlying neuronal death (Vosler et al., 2008). Calpain inhibitors have proven to be effective neuroprotectants in models of *status epilepticus* (Araujo et al., 2008; Wang et al., 2008). Recently, calpain I (μ-calpain) was shown to be responsible for triggering AIF release during excitotoxicity (Cao et al., 2007). Thus, calpain and AIF may be particularly important for neuronal death in seizure models in which caspases are not activated (Fujikawa et al., 2007; Wang et al., 2008; Zhao et al., 2010).

Bcl-2 FAMILY PROTEINS IN SEIZURE-INDUCED NEURONAL DEATH

In vivo delivery of Bcl-XL or Bcl-2 in rodents can prevent neuronal death following excitotoxin administration *in vivo* (Lawrence et al., 1996; Ju et al., 2008). Also, mice lacking another anti-apoptotic member Bcl-w were found to be more vulnerable to seizures and to develop significantly more hippocampal damage after *status epilepticus* (Murphy et al., 2007). Thus, anti-apoptotic Bcl-2 family members protect against seizure-induced neuronal death in animal models.

Among the multi-BH domain proteins, Bax has been shown to accumulate at mitochondria after seizures (Henshall et al., 2002) but we await *in vivo* functional studies. Some *in vitro* data support a role for Bax in glutamate-induced neuronal death (Xiang et al., 1998), but other studies have reported bax-deficient neurons are equally vulnerable to excitotoxicity (Dargusch et al., 2001; Cheung et al., 2005). Bak does not appear to promote neuronal death in adult brain (Fannjiang et al., 2003) and there are no *in vivo* data on the third multi-BH domain member, Bok.

At least six members of the BH3-only subfamily have been studied in seizure models, including each of the potently pro-apoptotic BH3-only subgroup (Engel et al., 2011). Bid is rapidly cleaved to its more active form after seizures (Henshall et al., 2001b; Li et al., 2006; Engel et al., 2010a). Bim and Puma are both up-regulated after seizures, accumulate in the hippocampus and bind anti-apoptotic Bcl-2 family proteins (Shinoda et al., 2004; Engel et al., 2010c; Murphy et al., 2010). Among the less potent members, there is evidence for activation of Bad after seizures (Henshall et al., 2002; Noh et al., 2006) while two other BH3-only proteins examined, Noxa and Hrk, were not induced (Korhonen et al., 2003; Engel et al., 2010c).

Signaling pathways linking seizures to activation of BH3-only proteins

How are the various BH3-only proteins activated by seizures? For Bad, the trigger is thought to be dephosphorylation by the calcium-regulated phosphatase calcineurin (Henshall et al., 2002). Puma upregulation after seizures is largely p53-mediated. Indeed, pharmacologic inhibition of p53 prevents Puma induction after *status epilepticus* and Puma is not up-regulated after seizures in p53-deficient mice (Engel et al., 2010c). The trigger for p53 accumulation after seizures may be DNA damage, which is an early consequence of seizures *in vivo* (Henshall et al., 1999; Lan et al., 2000), but other stimuli including raised intracellular calcium could be responsible (Sedarous et al., 2003).

Bim has several transcriptional control mechanisms (Biswas et al., 2007; Puthalakath et al., 2007; Concannon et al., 2010). The FoxO1/3a transcription factors were the first to be linked to Bim regulation after *status epilepticus* (Shinoda et al., 2004), but more recent work has implicated c-Jun N-terminal kinase (JNK; Murphy et al., 2010). Indeed, *in vivo* injection of the JNK inhibitor SP600125 fully blocked Bim induction after *status epilepticus* in mice (Murphy et al., 2010). Activation of JNK itself may occur via stimulation of the KA receptor subunit GluR6 recruiting post-synaptic density protein 95 (PSD-95) and mixed lineage kinases, which activate JNK (Savinainen et al., 2001; Li et al., 2010a). This signaling scaffold also mediates the degradation of Bcl-2 after seizures (Zhang et al., 2011). JNK can also be activated downstream of TNF receptor 1 and endoplasmic reticulum stress (Yang et al., 2006), which are both early events following seizures (Shinoda et al., 2003; Murphy et al., 2010). Last, *in vitro* data show the adenosine monophosphate-activated protein kinase (AMPK) mediates Bim-induced neuronal death during excitotoxicity (Concannon et al., 2010), although this has not been explored *in vivo*.

Caspase-8 has been implicated in the mechanism of Bid activation after seizures on the basis that caspase-8 pseudosubstrate inhibitors reduced Bid cleavage (Henshall et al., 2001b; Li et al., 2006). Whether this is downstream of death receptors is unknown. Bid can also be activated by calpain (Mandic et al., 2002) and in some models cleavage of Bid may not be needed for mitochondrial translocation and neuronal apoptosis (Konig et al., 2007).

In vivo evidence that BH3-only proteins regulate neuronal death after seizures

Seizure-induced neuronal death has recently been studied in animals lacking each of the potently pro-apoptotic BH3-only proteins (Engel et al., 2011). Mice deficient in Puma are the most protected against seizure-induced neuronal death, with seizure-damage reduced by over 70% (Engel et al., 2010c). Puma-deficient mice were also protected when the severity of *status epilepticus* was increased to produce a stronger necrotic component (Engel et al., 2010b). Seizure-damage in the hippocampus of Bim-deficient mice was reduced by ~45% in the same model (Murphy et al., 2010). In another study, however, bim−/− mice were not protected against intra-hippocampal KA-induced damage (Theofilas et al., 2009).

Seizure-induced hippocampal damage in Bid-deficient mice was not different to wild-type animals (Engel et al., 2010a). This was surprising because Bid-deficient mice were protected against stroke and traumatic brain injury, and bid−/− neurons are protected *in vitro* from glutamate excitotoxicity (Plesnila et al., 2001; Bermpohl et al., 2006). Of note, Puma-deficient mice are not protected against ischemic brain damage (Kuroki et al., 2009). This suggests there are differences in the contributions of BH3-only proteins to the patho-mechanisms of seizure versus ischemic damage as well as gaps between the relatedness of *in vitro* and *in vivo* models (Engel et al., 2011).

Bcl-2 family proteins in experimental epilepsy

Activation of apoptosis-associated signaling pathways also extends into the period of epileptogenesis and chronic recurrent seizures (i.e., epilepsy). Indeed, recent transcriptome profiling of rat brain

found changes to apoptosis-associated genes were associated with all stages of epileptogenesis (Okamoto et al., 2010). We know little, however, about the role of Bcl-2 family proteins in epilepsy development. Puma-deficient mice were found to develop fewer epileptic seizures than wild-type animals after *status epilepticus* (Engel et al., 2010c), but there are no data on Puma levels in wild-type epileptic mice. Also, the influence of Puma deficiency on epileptogenesis is complicated by the neuroprotection afforded by its absence during the initial precipitating injury. Studies of brief evoked seizures, which model some aspects of epileptic seizures, show expression changes for a small number of Bcl-2 family proteins but functional studies are needed to determine whether they are active during the process of epileptogenesis or after epileptic seizures (Engel and Henshall, 2009).

Bcl-2 family proteins in human epilepsy

The expression of several members of the Bcl-2 family has been examined in human hippocampus and neocortex samples resected from patients with TLE (Engel and Henshall, 2009). Overall, the patterns favor an adapted, anti-apoptotic state which may function to limit seizure-induced neuronal death; Bcl-2, Bcl-w, and Bcl-XL are all expressed at higher levels in TLE samples than controls and levels of Bim are lower (Henshall et al., 2000b; Shinoda et al., 2004; Murphy et al., 2007; Engel and Henshall, 2009). Modest upregulation of Bax has been reported in some TLE studies while no changes have been reported for Bad or Bid (Engel and Henshall, 2009). Bcl-2, Bcl-XL, and Bax may also be aberrantly expressed in focal cortical dysplasia, another common cause of pharmacoresistant epilepsy (Chamberlain and Prayson, 2008).

OTHER APOPTOSIS-ASSOCIATED SIGNALING PATHWAYS DURING EPILEPTOGENESIS AND IN CHRONIC EPILEPSY

Several other apoptosis-associated genes have been examined during epileptogenesis and in chronic epilepsy. Immunohistochemistry and enzyme assays show caspase-3 is active several days and even a week after *status epilepticus* (Narkilahti et al., 2003b; Weise et al., 2005). Other caspases have similarly been found to be cleaved and/or active during epileptogenesis and in epileptic brain in experimental models (Engel and Henshall, 2009). Over-expression and cleavage of caspases-1, 2, 3, 6, 7, and 9 have also been identified in resected human TLE tissue (Engel and Henshall, 2009).

NON-CELL DEATH FUNCTIONS OF APOPTOSIS-ASSOCIATED SIGNALING MOLECULES

It is recognized that several proteins involved in apoptosis have non-cell death-related biological functions (Galluzzi et al., 2008; Lamb and Hardwick, 2010). Caspase-1, a member of the non-apoptotic subgroup, is the enzyme responsible for interleukin-1β (IL-1β) production. An inflammatory cytokine, IL-1β is pro-convulsive, prolonging seizures, while inhibitors of IL-1β and caspase-1 have potent anti-convulsive effects (Vezzani et al., 2010). A role for caspase activation has also been demonstrated in microglial activation (Burguillos et al., 2011). Apoptotic caspases, including caspases-2 and 6, have been found to localize to the dendritic fields of the hippocampus in the wake of experimental status epilepticus (Narkilahti and Pitkanen, 2005; Narkilahti et al., 2007). Similar patterns of staining for caspases-2, 3, and 9 have been found in hippocampal tissue from TLE patients. This likely reflects roles for caspases in dendritic pruning and synaptic plasticity (D'amelio et al., 2010). Most recently, Bad, Bax, and caspase-3 have been proposed in long-term depression of synaptic transmission (Li et al., 2010c; Jiao and Li, 2011). These non-cell death functions are almost certainly of relevance to epilepsy. Only a single study has explored the effects of caspase inhibition on epilepsy, however, and results were negative (Narkilahti et al., 2003a). Nevertheless, if caspases and certain Bcl-2 family proteins influence the post-insult remodeling process and exert direct influences on excitability then they represent therapeutic targets of interest long-after the initial precipitating injury.

Other apoptosis-associated molecules have neuromodulatory effects. TNFα is known to promote AMPA receptor function (Stellwagen and Malenka, 2006) and Bcl-w has been associated with GABA receptor signaling (Murphy et al., 2007). It is likely further non-cell death functions will emerge for apoptosis-associated signaling molecules.

Taken together, studies on apoptosis-associated signaling in epilepsy have revealed a widespread role which spans contributions to early as well as late cell death and a variety of non-cell death-regulatory functions including seizure thresholds and processes which may impact on epileptogenesis and the chronic epileptic state.

The intracellular pathways involved in long-term neuronal cell death after seizures are illustrated in **Figure 2**.

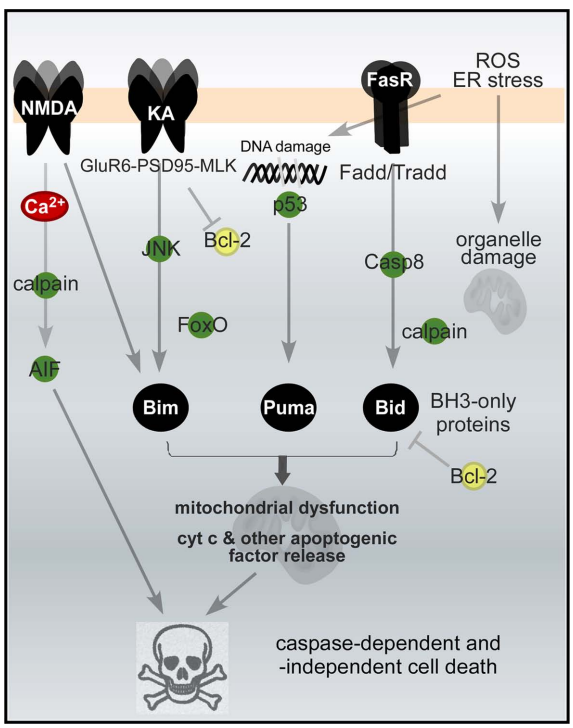

FIGURE 2 | Apoptosis-associated signaling pathways activated in neurons by seizures. Signaling pathways downstream of glutamate and Fas (death) receptors turn on BH3-only proteins of the Bcl-2 family, culminating in mitochondrial dysfunction and caspase-dependent and -independent cell death. The list is not complete and represents only some of the major pathways. Abbreviations: AIF, apoptosis-inducing factor; casp8, caspase-8; ER, endoplasmic reticulum; Fadd, Fas-associated death domain protein; FasR, Fas death receptor; JNK, Jun-terminal kinase; KA and NMDA, glutamate receptor subtypes; ROS, reactive oxygen species.

CONCLUSION

The activation of cell signaling pathways in response to acute seizures has detrimental long-term effects. Upregulation of IEGs induced by pathological overactivity results in plastic changes that alter synaptic functions and neuronal connectivity in susceptible brain areas. IEGs induction after seizures has been described to lead to neuronal death in several regions of the brain, including the hippocampus and the limbic system. These structures are among the most epileptogenic areas of the brain, and are crucially involved in learning and memory processes. In addition, studies on apoptosis-associated signaling in epilepsy have revealed a widespread role which spans contributions to early as well as late cell death and a variety of non-cell death-regulatory functions including seizure thresholds and processes which may impact on epileptogenesis and the chronic epileptic state. Thus, the activation of cell signaling pathways after seizures (markedly depending on the neuromodulatory action of catecholaminergic and serotonergic systems) has dramatic long-term consequences such as neuronal loss, behavioral changes and irreversible loss of function. Understanding these mechanisms remains a crucial issue to unravel the origins of epileptic behaviors and ultimately identify a cure for chronic epileptic disorders.

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Contribution of *Egr1/Zif268* to activity-dependent *Arc/Arg3.1* transcription in the dentate gyrus and area CA1 of the hippocampus

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Egr1, a member of the *Egr* family of transcription factors, and *Arc* are immediate early genes known to play major roles in synaptic plasticity and memory. Despite evidence that *Egr* family members can control *Arc* transcriptional regulation, demonstration of a selective role of *Egr1* alone is lacking. We investigated the extent to which activity-dependent *Arc* expression is dependent on *Egr1* by analyzing *Arc* mRNA expression using fluorescence *in situ* hybridization in the dorsal dentate gyrus and CA1 of wild-type (WT) and *Egr1* knockout mice. Following electroconvulsive shock, we found biphasic expression of *Arc* in area CA1 in mice, consisting in a rapid (30 min) and transient wave followed by a second late-phase of expression (8 h), and a single but prolonged wave of expression in the dentate gyrus. *Egr1* deficiency abolished the latest, but not the early wave of *Arc* expression in CA1, and curtailed that of the dentate gyrus. Since the early wave of *Arc* expression was not affected in *Egr1* mutant mice, we next analyzed behaviorally induced *Arc* expression patterns as an index of neural ensemble activation in the dentate gyrus and area CA1 of WT and *Egr1* mutant mice. Spatial exploration of novel or familiar environments induced in mice a single early and transient wave of *Arc* expression in the dentate gyrus and area CA1, which were not affected in *Egr1* mutant mice. Analyses of *Arc*-expressing cells revealed that exploration recruits similar size dentate gyrus and CA1 neural ensembles in WT and *Egr1* knockout mice. These findings suggest that hippocampal neural ensembles are normally activated immediately following spatial exploration in *Egr1* knockout mice, indicating normal hippocampal encoding of information. They also provide evidence that in condition of strong activation *Egr1* alone can control late-phases of activity-dependent *Arc* transcription in the dentate gyrus and area CA1 of the hippocampus.

Keywords: transcription factors, immediate early genes, hippocampus, spatial exploration, electroconvulsive shock, FISH, mutant mice

INTRODUCTION

Current hypotheses on the molecular mechanisms of learning and memory suggest that rapid regulation of gene programs and synthesis of new proteins leading to persistent synaptic modification constitute a key mechanism for the stabilization of long-term memory (Bruel-Jungerman et al., 2007 for a review). This genomic response includes a group of immediate early genes (IEGs) that encode two classes of proteins: nuclear transcription factors that regulate late-response genes, and proteins that directly modify synaptic function (Lanahan and Worley, 1998). Among these, *Egr1/Zif268* and *Arc/Arg3.1* are some of the best-characterized activity-regulated IEGs for their roles in synaptic plasticity and memory.

Egr1, a member of the early growth response (*Egr*) gene family, encodes a nuclear transcription factor that is rapidly and transiently induced during synaptic plasticity and in defined brain structures during memory consolidation or recall (reviewed in Davis et al., 2003; Knapska and Kaczmarek, 2004). Our previous studies in mutant mice have shown that *Egr1* is required for the expression of late-phase hippocampal long-term potentiation (LTP) and for the consolidation of several forms of long-term memory (Jones et al., 2001; Bozon et al., 2002, 2003; Davis et al., 2010). Like *Egr1*, *Arc* is required for synaptic plasticity and for several forms of long-term memory (Plath et al., 2006). Arc, however, is a direct effector protein at the synapse. Upon cell activation, *Arc* mRNA traffics to dendrites and accumulates at sites of synaptic activity, where it is locally translated (Link et al., 1995; Lyford et al., 1995; Steward et al., 1998) and plays important roles in homeostatic scaling of AMPA receptors and structural modifications at the synapse (Rial Verde et al., 2006; Shepherd et al., 2006; Messaoudi et al., 2007). The expression of *Arc* is regulated as an IEG (Pintchovski et al., 2009), but also as a late-response gene by a protein synthesis-dependent mechanism (Wallace et al., 1998; Li et al., 2005).

Despite the fact that Egr1 has a clear role in mediating gene expression required for learning and memory, the specific molecular mechanisms that are involved are poorly defined. Several potential Egr1 target genes have been suggested (Petersohn et al., 1995; James et al., 2005; Baumgartel et al., 2009), but few have been identified as potentially implicated in the *Egr1* mutant phenotype. Recently, Li et al. (2005) identified *Arc* as a direct target of the Egr family of transcription factors, showing Egr members can bind the *Arc* promoter *in vivo* after kainic acid-induced seizure and transactivate *Arc* through an Egr response element (ERE). In

this experiment however, Egr3 rather than Egr1 was found to be required for seizure-induced *Arc* expression in the dentate gyrus, whereas full induction of *Arc* after behavioral experience was suggested to depend upon both Egr1 and Egr3 (Li et al., 2005).

Here, we used *Egr1* knockout mice (*Egr1*$^{-/-}$) to investigate the extent to which activity-dependent *Arc* expression is dependent on Egr1. For this, we used fluorescence *in situ* hybridization (FISH; Guzowski et al., 1999), a sensitive method that has been extensively utilized to map neuronal networks activated by experience (reviewed in Miyashita et al., 2008). We examined *Arc* expression in wild-type (WT) and *Egr1*$^{-/-}$ mice following both electroconvulsive shock (ECS), a procedure known to induce robust expression of *Egr1* and *Arc* in the hippocampus, and following behavioral exploration of novel or familiar environments (Guzowski et al., 1999; Nakamura et al., 2010). Because activity-dependent *Arc* induction in hippocampus can be prolonged up to 8 h (Ramirez-Amaya et al., 2005), we analyzed the temporal dynamics of its expression over long time-intervals. Furthermore, we investigated *Arc* expression in both the dentate gyrus and area CA1, a region where *Egr1* is expressed constitutively in contrast to the dentate gyrus, to explore whether there is any structure-specificity in the ability of *Egr1* to control activity-dependent *Arc* expression. Finally, we used *Arc* FISH as an index of neural ensemble activation (Guzowski et al., 1999) to assess the proportion of dentate gyrus and CA1 cells activated by spatial exploration of novel and familiar environments in WT and *Egr1*$^{-/-}$ mice.

MATERIALS AND METHODS

SUBJECTS

Egr1$^{-/-}$ mice were generated using 129S2 ES cells injected into C57BL/6J blastocytes (Topilko et al., 1998) and backcrossed onto C57BL/6J background for 24 generations. Targeted inactivation of the *Egr1* gene involved insertion of a *lacZ*-neo cassette between the promoter and coding sequence and an additional frameshift mutation at the level of an *N*del restriction site that corresponds with the beginning of the DNA binding domain. As described previously (Jones et al., 2001), histochemical, physiological, and behavioral screening has shown that gross brain anatomy, basal hippocampal synaptic transmission and cell excitability, and general behavior and motor activity are normal in *Egr1*$^{-/-}$ mice. *In situ* hybridization studies also confirmed the complete absence of *Egr1* in mutant mice, while both constitutive and LTP-inducible expression of the *lacZ* gene in the *Egr1*$^{-/-}$ was comparable to that of the *Egr1* gene in WT mice, suggesting that signaling events upstream of Egr1 transcription are not affected in the mice. WT and *Egr1*$^{-/-}$ littermate male mice (9–14 months old) used in this study were generated by crossing heterozygous *Egr1*$^{+/-}$ mice to obtain progeny in which male siblings were either of a mutant or WT genotype, as before (Jones et al., 2001; Bozon et al., 2003). Mice were housed in a temperature and light-controlled colony room (12 h light/dark cycle) in groups of 4/5 with food and water *ad libitum*. The genotype was verified by PCR on tail DNA. All experiments were conducted during the light phase. All efforts were made to minimize the number of animals and their suffering throughout the experiments. Experiments were performed in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and the French National Committee (87/848).

APPARATUS, BEHAVIOR, AND TISSUE COLLECTION

Electroconvulsive shock was administered via ear-clip electrodes using a constant-current generator. Eighty-four mice were used in this experiment. Mice were anesthetized lightly with fluothane and a single 200 V shock was delivered for 2 s. Immediately following the shock, mice displayed tonic–clonic seizures and were placed back in their home cages before being sacrificed at delays of 5 min, 30 min, 4 h, 6 h, or 8 h after ECS (WT: $n = 6, 12, 6, 6, 5$; *Egr1*$^{-/-}$: $n = 6, 6, 6, 6, 7$, respectively). WT and *Egr1*$^{-/-}$ cage control (CC) mice were killed at the same time of day as experimental mice (WT: $n = 11$; *Egr1*$^{-/-}$: $n = 7$).

In behavioral experiments, mice were first handled twice daily for 2 days before training. The next day (D1), mice in the "familiar environment" condition were placed in an open-field arena (45 cm × 45 cm × 30 cm) with wood shavings on the floor, positioned on a table (120 cm high) in an experimental room containing several distal cues. They were allowed to explore the arena for 10 min before being placed back in their home cages. This was repeated twice daily for 3 days (D1–D3), and once more on D4. Mice in the "novel environment" condition were allowed to explore the open-field arena (for 10 min) only once. Behavior of the animals was monitored automatically via a camera above the arena and using the ANY-maze program (Stoelting). Subgroups of WT and *Egr1*$^{-/-}$ mice from both experimental conditions were killed 30 min or 8 h after the end of exploration. Both WT and *Egr1*$^{-/-}$ mice displayed extensive exploration of the open-field arena, as evidenced by their multiple crossings of the 25 virtual square sectors in the arena (mice of both genotypes entered each square at least eight times on average). Fifty-four mice were used in the behavioral experiment (WT and *Egr1*$^{-/-}$, respectively: familiar 30 min $n = 4$ and 4; novel 30 min $n = 4$ and 4; familiar 8 h $n = 5$ and 4; novel 8 h $n = 7$ and 7). Undisturbed WT and *Egr1*$^{-/-}$ CC mice were killed at the same time of day as experimental mice (WT: $n = 8$; *Egr1*$^{-/-}$: $n = 7$).

FLUORESCENT *IN SITU* HYBRIDIZATION

After cervical dislocation, the brains were removed rapidly, quick-frozen in isopentane (−40°C), and stored at −80°C until being sectioned on a cryostat. Twenty-micrometer-thick sections were mounted on slides such that the groups were distributed on the slides in a pseudorandom manner. For normalization purposes, each slide contained a WT CC and a WT section from the 30-min delay. Slides were air dried and stored frozen at −80°C until use. A commercial transcription kit and premixed RNA labeling nucleotide mixes containing digoxigenin-labeled UTP (Roche, France) were used to generate cRNA riboprobes. Riboprobes were purified on Mini Quick Spin RNA columns (G-50, Roche). The yield and integrity of riboprobes was confirmed by gel electrophoresis. The plasmid used to generate the *Arc* antisense and sense riboprobes contained a nearly full-length cDNA (~3 kbp, courtesy of Dr. D. Kuhl) of the *Arc* transcript (Lyford et al., 1995). Slide-mounted sections were fixed in 4% buffered paraformaldehyde, treated with 0.75% acetic anhydride/1.1% triethanolamine (Roth), then with 50% acetone/50% methanol (Roth), and equilibrated in 2× SSC (Roth). Slides were incubated in 1× hybridization buffer, consisting of 4× SSC, 50% formamide (Roth), 1× Denhardt's solution (Sigma), 10% dextran-sulfate (Roth), 0.05% fish sperm DNA (Roche), 0.025% yeast tRNA (Roche), for 30 min at room temperature.

Riboprobe (100 ng) was diluted to 100 µl in the hybridization buffer, heat denatured, chilled on ice, and then added to each slide. Hybridization was carried out at 55°C for 16 h. Slides were washed to a final stringency of 0.5× SSC at 56°C; these washes included an earlier wash step at 37°C in 2× SSC with RNase A (10 µg/ml, Sigma). Endogenous peroxidase activity was quenched with 2% H_2O_2 in 1× SSC, slides were blocked (blocking reagent, Perkin-Elmer), and incubated with an appropriate horseradish peroxidase (HRP)-antibody conjugate (Roche) for 2 h. Slides were washed three times in Tris-buffered saline (with 0.05% Tween-20), and the conjugate was detected using TSA-direct system (Perkin-Elmer). Nuclei were stained with Sytox green (Invitrogen). Slides were coverslipped with antifade media (Fluoromount, Southern Biotech).

IMAGE ACQUISITION AND ANALYSIS

All analyses were run blind to the genotype and experimental conditions. Images were acquired with a laser confocal microscope (argon–krypton laser; Zeiss MRC1024ES; Jena, Germany). Cross-over fluorescence could be ruled out as spectra of the fluorochromes did not overlap. Each optical section was averaged three times. Photomultiplier tube assignments, pinhole size, and contrast values were kept constant for each brain region within a slide. For each experiment, images were acquired from sections on two or three slides, ~1.7 mm posterior to bregma. The free software ImageJ (http://rsb.info.nih.gov/ij) was used to analyze captured images. Only putative neurons were included in the analyses. Putative glial-cell nuclei were identified based on their small size (~5 µm in diameter) and bright, uniform nuclear counterstaining (Guzowski et al., 1999); these cells never expressed *Arc*.

Two types of analyses were performed: counting the number of cells expressing *Arc* mRNA signal and optical density measurements, which allowed us to measure differences in signal intensity even in conditions of unchanged number of labeled cells. In the ECS experiment, only optical density analysis could be done, as 95–100% of the neurons expressed *Arc* after this strong stimulus, as previously described in the rat (Guzowski et al., 1999), making it impracticable to obtain cell counts. In ECS experiments, for each mouse, two to three z-stacks were collected from the CA1 and dentate gyrus cell layers (each from a different slide). Z-stacks consisted of the three middle planes of the brain section (1 µm optical thickness/plane), collected by using a 60× oil immersion objective. The field of view using this objective was 205 µm × 136 µm. For image analysis, the three stacks were "flattened" into one image, using a projection method taking into account only the highest (maximum) pixel value of the three stacks. The area of interest was then selected by visualizing only the color channel containing Sytox labeling (cell nuclei) and using the "Lasso" tool. The mean optical density value in the region of interest was determined for the color channel containing the CY3 labeling (*Arc* mRNA staining). Threshold levels were determined on the basis of WT CC images, where *Arc* expression was limited to a few scattered cells. The threshold value was held constant for each batch of slides processed together. Finally, values for experimental mice were normalized to those of WT caged controls (0%) and to WT mice with the 30-min delay post-ECS (100%). This normalization procedure minimizes artifact caused by slide-to-slide variation in signal intensity and background.

In behavioral experiments and for *Arc* expression in area CA1, z-stacks consisting of 1 µm-thick optical sections were acquired with a 60× oil objective. To estimate the proportion of *Arc*-positive neurons, 184 neurons per mouse on average were counted (from four non-overlapping z-stacks from two slides). First, neuronal nuclei present in the median planes (representing 20% of the stack thickness) were identified and outlined. Nuclei were then characterized for the presence of *Arc* cytoplasmic labeling (only nuclei with labeling in very close proximity were considered as positive), and the results expressed as a percentage of total nuclei analyzed per stack. The median planes criterion reduced the likelihood of analyzing partial nuclei, which could yield false negative results (West, 1993; Guzowski et al., 1999). We also estimated the amount of *Arc* mRNA expression by measuring optical density in CA1 pyramidal layer, *stratum radiatum* and *stratum oriens* (containing apical and basal dendrites, respectively), following the method described above for the ECS experiment (four non-overlapping z-stacks from two slides per animal were used).

In granule cells of the dentate gyrus, *Arc* staining was extremely sparse, thus to avoid sampling bias we imaged the entire dentate gyrus (four to six sections/mouse, left and right side from two to three slides; Vazdarjanova et al., 2006). Each dentate gyrus image was reconstructed from overlapping 10× z-stacks by using the shape of cell groups as landmarks. Cells exhibiting *Arc* cytoplasmic labeling were counted. The area of each section was measured and used to estimate the total number of neurons using a correction factor that represented total neurons per square micrometer. This factor was derived from 10 z-stacks from six different mice collected at 60× magnification (granule cell counts did not vary significantly across these 10 slices). Data from the upper and lower blades of the dentate gyrus were analyzed separately. The results were expressed as percent of estimated total cell number.

STATISTICS

Because of small sample sizes, effects of genotype and treatment (time after ECS or behavioral testing condition and delay) on Arc mRNA expression were evaluated by non-parametric Kruskal–Wallis test. When the main effect was significant at $p \leq 0.05$, additional comparisons between groups were conducted with the non-parametric Mann–Whitney *U*-test. Behavioral data were obtained on a larger sample, they were therefore analyzed by ANOVA, followed by Fisher's LSD *post hoc* tests, when appropriate. Correlations between behavioral parameters and *Arc* mRNA expression in each structure were analyzed by the non-parametric Spearman's rank correlation test.

RESULTS

BASAL *ARC* mRNA EXPRESSION

In caged control mice, a small percentage of CA1 pyramidal cells and dentate granule cells (DGCs) exhibited *Arc* expression in WT mice (9.8 and 0.32%, respectively; **Figures 1–4**). A similar profile was observed in *Egr1*$^{-/-}$ mice (7.5 and 0.40% in CA1 and dentate gyrus, respectively; **Figures 1–4**), with no significant difference between genotypes (CA1: $p = 0.73$; dentate gyrus: $p = 0.49$).

ARC TRANSCRIPTION INDUCED BY ELECTROCONVULSIVE SHOCK

In WT mice, ECS-induced robust transcription of *Arc* mRNA in CA1 and dentate gyrus neurons, compared with CC mice (**Figures 1 and 2**). The kinetics of ECS-induced *Arc* mRNA expression in the cytoplasm

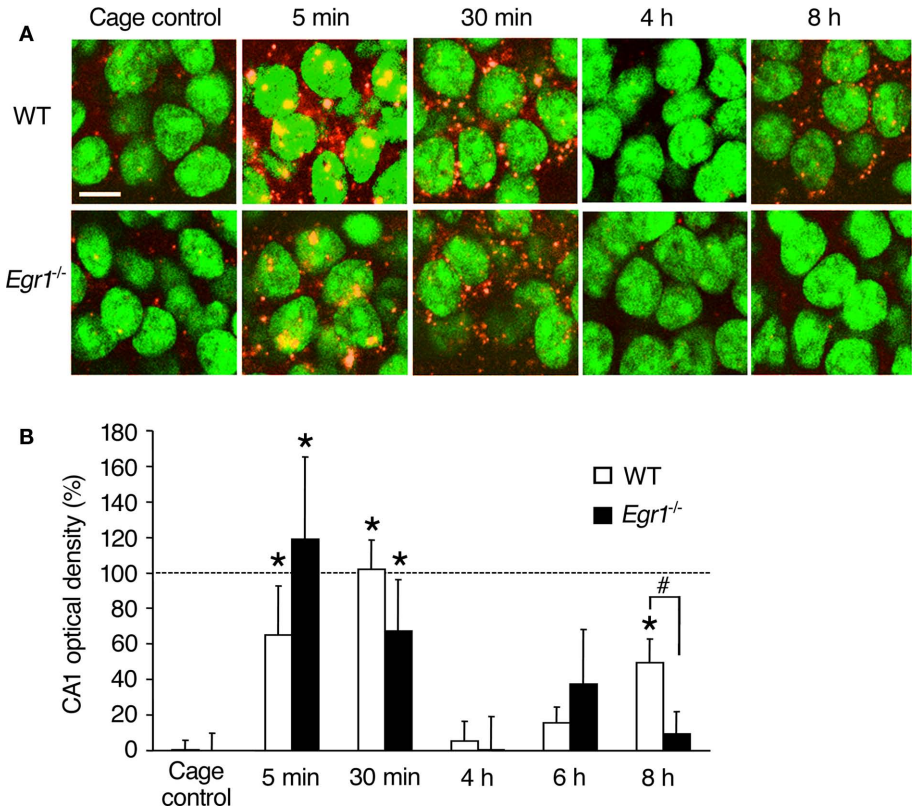

FIGURE 1 | *Arc* mRNA expression after ECS in area CA1 of WT and *Egr1*$^{-/-}$ mice (cage control, CC). (A) Representative images of *Arc* mRNA detection using FISH in CA1 pyramidal neurons in CC mice and at different times after ECS. Cell nuclei appear in green (Sytox) and *Arc* mRNA signal in red (or yellow in the nucleus). *Arc* mRNA is intra-nuclear 5 min after ECS and mainly cytoplasmic 30 min after ECS. Nearly 100% of CA1 neurons express *Arc* mRNA after ECS. Scale bar, 10 µm. **(B)** Optical density of *Arc* mRNA signal in CA1. After initial *Arc* mRNA induction, the level of expression returns to baseline by 4–6 h, and a second wave is observed 8 h after ECS. This second wave is not observed in *Egr1*$^{-/-}$ mice. Error bars in this and subsequent figures indicate SEM. *Different from CCs of the same genotype, at least $p < 0.05$. #Difference between genotypes, $p < 0.05$. Dashed line indicates 100% (normalized to WT 30 min).

was of different appearance in CA1 and the dentate gyrus. In area CA1 of WT mice, a clear increase in intra-nuclear *Arc* expression was observed 5 min after ECS, followed by cytoplasmic expression at 30 min (**Figure 1**). *Arc* expression then returned to control levels at 4 and 6 h and a second wave of expression was observed at 8 h. Overall Kruskal–Wallis analysis showed a significant effect of ECS and/or genotype on *Arc* mRNA expression ($p = 0.0004$). Subsequent Mann–Whitney *U*-tests showed that in CA1 of WT mice, *Arc* mRNA expression was elevated 5 and 30 min after ECS compared with CC mice (5 min: $p = 0.005$; 30 min: $p < 0.00001$). Optical density values were not different from that of controls at 4 and 6 h following ECS, but again a significant increase was observed during the second wave at 8 h ($p = 0.002$), reaching ~50% of that observed at 30 min. In *Egr1*$^{-/-}$ mice, *Arc* expression was also significantly elevated at 5 and 30 min compared with CC mice (5 min: $p = 0.02$; 30 min: $p = 0.04$). In contrast to the WT, however, no second wave of elevated *Arc* expression at 8 h was observed in CA1 of *Egr1*$^{-/-}$ mice ($p = 0.28$). Moreover, between-group comparison showed that CA1 *Arc* mRNA expression 8 h after ECS was significantly lower in *Egr1*$^{-/-}$ than in WT mice ($p = 0.04$).

In the dentate gyrus of WT mice (upper blade), intra-nuclear labeling was observed in only few cells 5 min after ECS, but a large cytoplasmic increase in *Arc* mRNA expression was observed from 30 min up to 8 h post-ECS (**Figure 2**). Overall, there was a significant effect of ECS and/or genotype (Kruskal–Wallis $p < 0.00001$). In contrast to CA1, the increase in *Arc* mRNA expression in the dentate gyrus of WT mice followed a single wave and was significantly elevated compared to CC mice at all time points (5 min: $p = 0.01$; 30 min: $p < 0.00001$; 4 h: $p = 0.0005$; 6 h: $p = 0.0005$; 8 h: $p = 0.004$). In *Egr1*$^{-/-}$ mice, *Arc* mRNA was induced to a similar extent than in WT mice from 30 min to 6 h post-ECS (5 min: $p = 0.10$; 30 min: $p = 0.001$; 4 h: $p = 0.001$; 6 h: $p = 0.01$; **Figure 1**) with no difference between genotypes at any of these time points (all $p > 0.05$). However, no significant increase was detected in *Egr1*$^{-/-}$ at 8 h ($p = 0.32$). Moreover, comparison between groups showed a clear trend for *Arc* expression to be lower in *Egr1*$^{-/-}$ than in WT mice ($p = 0.06$), even if the level of ECS-induced expression in the dentate gyrus of WT mice represented only ~5% of the initial increase observed at 30 min.

ARC TRANSCRIPTION INDUCED BY SPATIAL EXPLORATION IN AN OPEN-FIELD

Analysis of ECS-induced *Arc* mRNA expression indicated that long-term, but not short-term, expression was deficient in CA1 and the dentate gyrus of *Egr1*$^{-/-}$ mice. Thus, we next focused on the

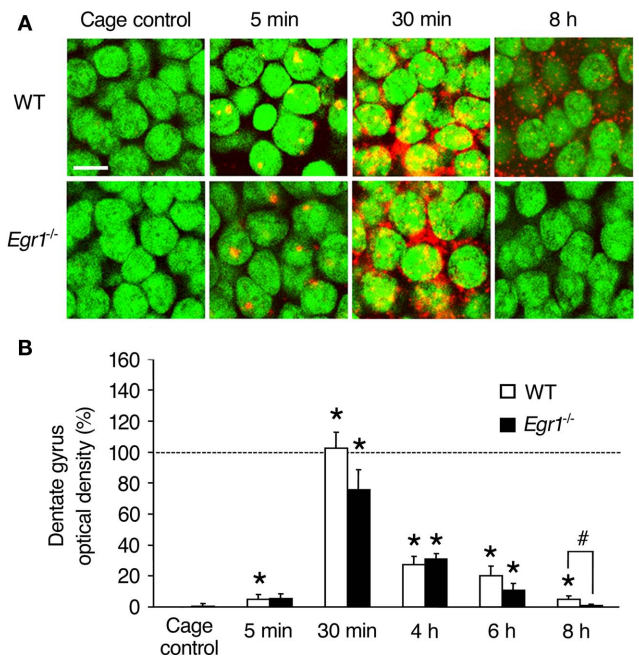

FIGURE 2 | *Arc* mRNA expression after ECS in the dentate gyrus (DG) of WT and *Egr1*$^{-/-}$ mice. (A) Representative images of *Arc* mRNA detection in granule cells of the dentate gyrus in cage control mice and at different times after ECS. Cell nuclei appear in green (Sytox) and *Arc* mRNA signal in red (or yellow in the nucleus). *Arc* mRNA is intra-nuclear 5 min after ECS and mainly cytoplasmic 30 min after ECS. Scale bar, 10 μm. **(B)** Optical density of *Arc* mRNA signal in dentate gyrus. ECS-induced *Arc* mRNA expression is maintained for at least 8 h in WT mice, but decays more rapidly in *Egr1*$^{-/-}$ mice and is not significantly above the cage control baseline level after 6 h. *Different from cage controls of the same genotype, at least $p < 0.05$. #Difference between genotypes, $p = 0.06$. Dashed line indicates 100% (normalized to WT 30 min).

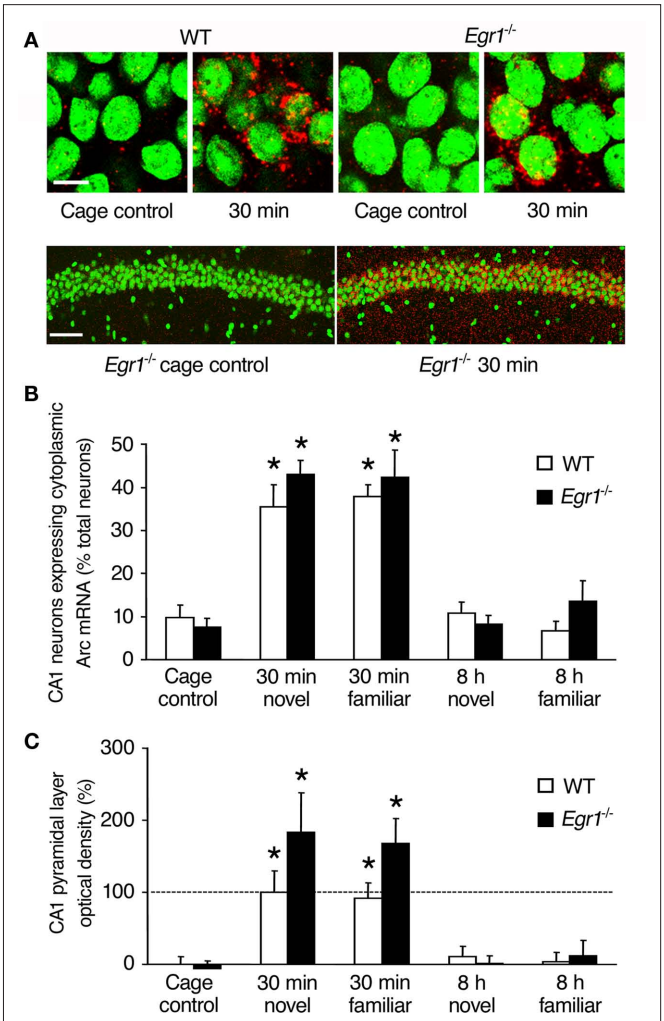

FIGURE 3 | *Arc* mRNA expression after spatial exploration in CA1 of WT and *Egr1*$^{-/-}$ mice. (A) Representative images from WT and *Egr1*$^{-/-}$ cage control mice and 30 min after exploration of a novel or familiar environment. Scale bar, 10 μm. Below are sample lower definition images of Arc mRNA expression in CA1 from two *Egr1*$^{-/-}$ mice (left: cage control; right: 30 min after spatial exploration). Scale bar, 100 μm. Cell nuclei appear in green (Sytox) and *Arc* mRNA signal in red (or yellow in the nucleus). **(B)** Proportion of the neurons expressing *Arc* mRNA in the pyramidal layer after exploration of a novel or familiar environment. **(C)** Normalized optical density of *Arc* mRNA signal in the pyramidal layer. *Arc* mRNA expression was similarly induced in WT and *Egr1*$^{-/-}$ mice 30 min, but not 8 h following exploration. *Different from cage controls of the same genotype, at least $p < 0.05$. Dashed line indicates 100% (normalized to WT 30 min).

short-term (30 min) and long-term (8 h) time points to examine behaviorally induced *Arc* mRNA expression in WT and *Egr1* mice. Two experimental conditions were used: exposure to a novel or to a familiar environment (see Materials and Methods).

Behaviorally, there was evidence for habituation in the familiar environment, as shown by the decrease in locomotor activity across the six sessions (trial 1: 30.7 m, trial 6: 20.9 m; session effect $p < 0.0001$). Moreover, during the session preceding sacrifice, mice in the familiar environment ambulated ~20% less than mice exploring for the first time the novel environment (25.3 vs. 29.0 m, respectively; $p = 0.03$). Although *Egr1*$^{-/-}$ mice habituated similarly to the WT, a genotype effect was observed: overall locomotion in *Egr1*$^{-/-}$ mice was lower than in WT mice, with an average of ~20% less distance moved than WT mice (genotype effect: $p = 0.009$). As reported previously in rats (Guzowski et al., 1999; Chawla et al., 2005; Ramirez-Amaya et al., 2005; Vazdarjanova et al., 2006), open-field exploration-induced a clear increase in *Arc* mRNA expression in both CA1 pyramidal cells and DGCs of WT mice, compared with the control groups (**Figures 3 and 4**).

In area CA1, we first estimated the proportion of neurons expressing cytoplasmic *Arc* mRNA at each time point (**Figure 3**). Overall Kruskal–Wallis analysis showed a significant effect of genotype, of open-field exploration and/or novelty of the environment on *Arc* expression ($p = 0.0001$; **Figure 3B**). Subsequent Mann–Whitney *U*-tests showed that *Arc* mRNA expression in WT mice was significantly elevated 30 min after exploration compared with CC mice, both following exposure to the novel and familiar environments (novel: $p = 0.02$; familiar: $p = 0.006$). In contrast to ECS-induced expression, there was no late wave of increased *Arc* expression in area CA1 of WT mice 8 h following behavioral exploration of either the novel or familiar environment. In area CA1 of *Egr1*$^{-/-}$ mice, a similar pattern of behaviorally induced *Arc* expression to that of WT mice was observed: *Arc* mRNA expression was significantly increased 30 min, but not 8 h, following

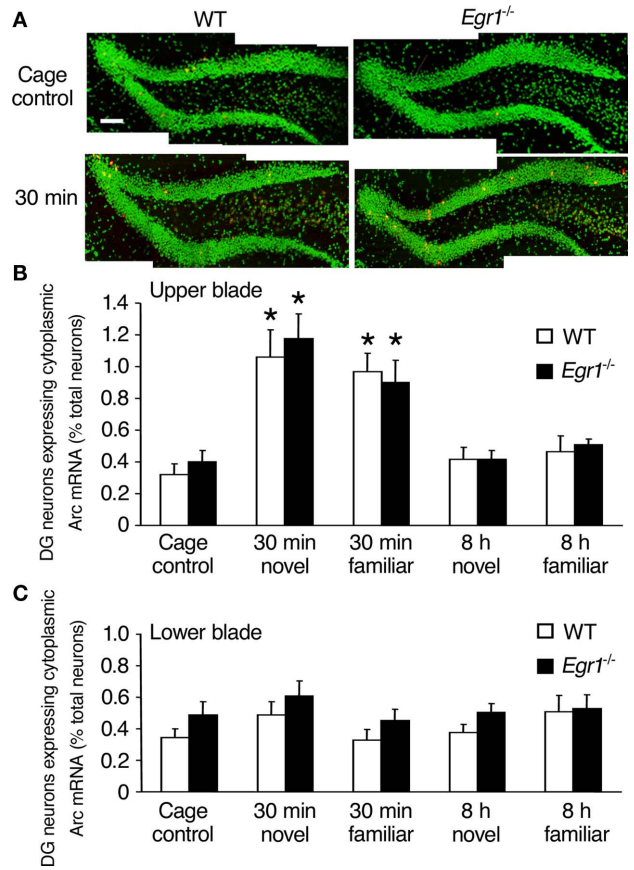

FIGURE 4 | *Arc* mRNA expression after spatial exploration in the dentate gyrus (DG) of WT and *Egr1*$^{-/-}$ mice. (A) Representative images of dentate gyrus sections from WT and *Egr1*$^{-/-}$ cage control mice and 30 min after exploration of a novel or familiar environment. Scale bar, 300 µm. Cell nuclei appear in green (Sytox) and *Arc* mRNA signal in red (or yellow in the nucleus). **(B,C)** Proportion of neurons expressing *Arc* mRNA in the upper **(B)** and the lower blade **(C)** of the dentate gyrus after exploration of a novel or familiar environment. In the upper blade, *Arc* mRNA expression was similarly induced in WT and *Egr1*$^{-/-}$ mice 30 min, but not 8 h following exploration of a novel or a familiar environment. *Different from cage controls of the same genotype, at least $p < 0.05$.

exploration in both environmental conditions, compared with CC mice (30 min novel: $p = 0.008$; familiar: $p = 0.008$). Comparison of matching groups of WT and *Egr1*$^{-/-}$ mice showed that the proportion of *Arc*-positive cells in CA1 was similar at all time points and conditions. Moreover, novelty of the environment had no specific effect compared with familiar environment in CA1, regardless of genotype or delay.

Once established that *Egr1* deficiency did not affect the proportion of neurons expressing *Arc* mRNA in CA1, neither at the basal condition nor after exploration, we estimated the amount of transcript expression by measuring optical densities in the pyramidal layer and in *stratum radiatum* and *stratum oriens*. Similarly to our results on the proportion of *Arc*-expressing neurons, optical density values 30 min after exploration were significantly increased in the pyramidal layer (**Figure 3C**) and *stratum radiatum* and *oriens* (data not shown) in both WT and *Egr1*$^{-/-}$ mice and in response to exposure to both the novel and familiar environments (Mann–Whitney *U*-test, all $p < 0.05$ compared with CC). The increase was slightly larger in *Egr1*$^{-/-}$ mice than in WT mice but this was not significant (all $p > 0.05$). Again, there was no significant increase in *Arc* expression at the 8-h time point in any of the different CA1 layers, genotypes or behavioral conditions (all $p > 0.05$). Direct comparison of matching groups of WT and *Egr1*$^{-/-}$ mice showed that *Arc* expression in area CA1 was similar for all time points and conditions, and there was no specific effect of spatial exploration of the novel vs. familiar environment, regardless of genotype or delay. Analyses of correlations between behavioral parameters (locomotor activity, immobility, percent time spent near the wall) and *Arc* mRNA expression levels (proportion of *Arc*-positive cells, optical density values) revealed no significant correlation at either delay (all $p > 0.05$).

In the dentate gyrus, *Arc* mRNA expression following exploration increased only in the upper blade and the apex (together referred to as upper blade hereafter), but not in the lower blade (**Figure 4**), as previously reported in rats (Chawla et al., 2005). This was confirmed by overall Kruskal–Wallis analysis showing statistically significant effects of genotype, open-field exploration and/or novelty on *Arc* mRNA expression in the upper blade ($p = 0.0001$), but not the lower blade ($p = 0.36$). In the upper blade of the dentate gyrus of WT mice, *Arc* mRNA expression was significantly increased compared with CC mice 30 min after exploration of both the novel and familiar environments (novel: $p = 0.01$; familiar: $p = 0.006$; **Figure 4**). In neither behavioral conditions was elevated *Arc* expression maintained at 8 h (all $p > 0.05$). In *Egr1*$^{-/-}$ mice, the level of *Arc* mRNA expression in the upper blade of the dentate gyrus was also significantly increased 30 min following exploration of both types of environments (novel: $p = 0.008$; familiar: $p = 0.02$), but not 8 h thereafter ($p > 0.05$ in each case). Direct comparison of matching groups of WT and *Egr1*$^{-/-}$ mice showed that *Arc* mRNA expression in the dentate gyrus was similar for all time points and conditions, and exploration of the novel or familiar environment had a similar effect regardless of genotype or delay (all $p > 0.05$). As in CA1, correlational analyses between behavioral parameters (locomotor activity, immobility, percent time spent near the wall) and *Arc* mRNA expression levels revealed no significant correlation at either delay (all $p > 0.05$).

DISCUSSION

Two major findings of this study are that: (1) *Egr1* is not necessary for *Arc* induction in the regions of the dorsal dentate gyrus and area CA1 analyzed, shortly (30 min) after neuronal activation; (2) *Egr1* plays a significant role in activity-dependent delayed expression of *Arc* (8 h). Specifically, ECS-induced biphasic expression of *Arc* mRNA in area CA1 of WT mice, with a first wave of cytoplasmic expression 30 min after ECS and, after a decline to baseline by 4–6 h, a second, later phase of *Arc* expression in CA1 pyramidal cells at 8 h. We found that this late-phase of *Arc* expression in CA1 was completely absent in *Egr1*$^{-/-}$ mice. In DGCs, ECS rapidly (5 min) induced a single and prolonged wave of *Arc* mRNA expression, which decayed progressively but was still visible 8 h after ECS. With *Egr1*$^{-/-}$ mice, this wave of *Arc* expression in DGCs was curtailed in its longer-lasting phase (between 6 and 8 h). In the report by Li et al. (2005), *Egr3*, but not *Egr1*, was shown to be required for *Arc* expression in the dentate gyrus 4 h after kainic acid-induced seizures. Here, consistent with this result we did not

observe a significant reduction in *Arc* expression 4 h after seizure in the dentate gyrus of *Egr1*$^{-/-}$ mice; however, examining longer time points following ECS, a later phase of *Arc* induction occurring at 8 h was not observed in hippocampal sub-regions in *Egr1*$^{-/-}$ mice, providing evidence that the *Egr1* member alone can control the later phases of activity-dependent *Arc* gene transcription. The fact that *Egr1* only affects the late induction of *Arc* is in agreement with the proposed role of this transcription factor in the second wave of gene expression driven by activity since *Egr1* itself is induced by activity.

Short-term activity-dependent transcription of *Arc* is regulated as an IEG by recruitment of phosphorylated transcription factors acting at least in part via SRE and a Zeste-like response elements (Pintchovski et al., 2009). This early phase does not require protein synthesis (Wallace et al., 1998; Li et al., 2005). The early phase of *Arc* expression observed in WT mice is therefore probably due to post-translational modification of pre-existing transcription factors. Our results showing that short-term *Arc* transactivation is normal in *Egr1*$^{-/-}$ mice both after seizure and after spatial exploration suggest that Egr1 is dispensable for this early phase, even in CA1 where Egr1 is constitutively expressed. In contrast, our results strengthen the idea that the late-phase of *Arc* transcription requires transactivation via synthesized transcription factors, among which Egr1. Egr1 protein binds to a cognate GC-rich consensus DNA binding motif, the ERE. An ERE consensus sequence was identified proximal to the transcription start site of the *Arc* promoter (Li et al., 2005). In the dentate gyrus, Egr1 behaves as an inducible transcription factor with little, if any, constitutive expression (e.g., Beckmann and Wilce, 1997; French et al., 2001). It is therefore likely that activity-dependent *Egr1* transcription, translation and binding to the ERE is required for initiating the late component of activity-dependent *Arc* transcriptional regulation. In area CA1, where basal expression of Egr1 is relatively high, its role in mediating the late wave of *Arc* induction could be via the same mechanisms and/or via post-translational modification of the existing Egr1 proteins leading to increased binding activity to the ERE. Further experiments would be required to determine whether the molecular mechanisms leading to Egr1-dependent transcription of *Arc* are similar in both hippocampal sub-regions.

Surprisingly, and in contrast to what has been reported in CA1 and dentate gyrus in rats 8 h after exploration of a novel environment (Ramirez-Amaya et al., 2005), the present experiment showed that WT mice did not express levels of *Arc* mRNA higher than caged controls at this late time point. This disparity between previous rat studies and our own likely reflects species differences. Notably, however, our *Arc* expression data in WT mice after ECS, both in CA1 and dentate gyrus, show a very similar pattern and kinetics of expression to that reported after spatial exploration in rats, with reactivation of CA1 *Arc* expression 8 h after ECS and prolonged expression up to 8 h in the dentate gyrus. This indicates that mouse hippocampal neurons have the intrinsic capacity for expressing activity-dependent late-phase of *Arc* transcriptional regulation. The reason for the absence of late expression of *Arc* after spatial exploration in mice compared to rats is at present unclear. One obvious possibility is that stronger initial synaptic activation is necessary in mice for the expression of the second wave of *Arc* in CA1 and for maintaining *Arc* expression over 8 h in the dentate gyrus. A stronger or more prolonged activation could possibly be achieved by using more salient behavioral paradigms such as contextual fear conditioning or spatial learning in the water maze. Alternatively, it remains possible that different molecular mechanisms are engaged in mice and rats for stabilizing neuronal ensembles activated during spatial exploration or that the process of stabilization of the activated neuronal ensembles is achieved more quickly in mice than in rats.

Besides this substantial difference between the two rodent species, several other features of *Arc* expression in hippocampal neurons following spatial exploration appear similar in mice and rats. First, similar to the findings in rats (Chawla et al., 2005; Ramirez-Amaya et al., 2005), we found that Arc-expressing neurons in CA1 were relatively homogeneously scattered along the pyramidal layer, whereas in the dentate gyrus *Arc* was induced in the upper blade and genu of the hilus, but virtually absent in the lower blade. Second, comparison between the present results in WT mice with previous reports in rats (Guzowski et al., 1999; Vazdarjanova et al., 2002; Ramirez-Amaya et al., 2005) suggests that spatial exploration activates neural ensembles of similar size in both species. Activity-dependent *Arc* expression is believed to be a reliable marker of neural ensembles activated by experience (Guzowski et al., 1999, 2006). In dorsal CA1, we found that *Arc* was expressed in 35.5% of the neurons 30 min after exploration, nearly identical to the proportion reported in rats (e.g., 38% in Vazdarjanova et al., 2002). In the dentate gyrus, the population of DGCs expressing *Arc* in response to spatial exploration is usually much smaller than in CA1 (Guzowski et al., 1999; Ramirez-Amaya et al., 2005; Vazdarjanova et al., 2006). The proportion of *Arc*-positive cells in the upper blade of the dentate gyrus in our experiment seemed slightly smaller (1.1%) than found in a comparable study in rats (2.4%; Chawla et al., 2005). In our mice, however, the proportion of *Arc*-positive neurons was also lower at the basal state than in rats (0.3 vs. 0.6% in the study of Chawla et al., 2005). Therefore, the ratio of *Arc*-expressing neurons after exploration to the basal state is closely similar (~4×) in both species. In all, comparison of estimated numbers of activated cells suggests that spatial exploration recruits similar size dentate gyrus and CA1 neural ensembles in rats and mice. Moreover, our finding of similar size *Arc*-positive neural ensembles activated in both the dentate gyrus and CA1 between the two genotypes suggests normal neuronal encoding of information in the two hippocampal areas of *Egr1*$^{-/-}$ mice.

We also examined the contribution of relative familiarity of the environment. In WT mice, we found no difference in the proportion of *Arc*-positive cells after exploring novel or familiar environments, neither in area CA1 (35.5 vs. 38.0%, respectively) nor in the dentate gyrus (1.06 vs.1.17%, respectively), indicating that *Arc* expression is not directly linked to novelty. Of note, *Arc* transactivation was unaltered in *Egr1*$^{-/-}$ mice despite the fact that the mice displayed lower scores of locomotor activity compared to WT mice (by 20%). Moreover, locomotor activity also decreased by 20% in the familiar environment across successive sessions of habituation. Despite this, however, mice of both genotypes showed similar numbers of *Arc*-positive neurons in the familiar and novel environments. Thus, although this could be due to the fact that both genotypes displayed important locomotor activity, it points to the possibility that locomotor activity *per se* might not be the most critical factor determining the extent of the neural ensembles activated by experience

in this paradigm. Our results are in line with those reported in rats (Guzowski et al., 2006: CA1: 37 vs. ~39%; Chawla et al., 2005: dentate gyrus: 1.9 vs. 1.7%), showing that exploration-induced *Arc* transcription in CA1 and dentate gyrus does not habituate with repeated exploration of the same environment when exposures are separated by 24 h. Our study thus confirms in mice that *Arc* gene transcription is coupled to neural activation and does not distinguish between neural activity associated with new learning or memory retrieval (Miyashita et al., 2008).

With *Egr1*$^{-/-}$ mice, the patterns of *Arc* induction in the dentate gyrus and in area CA1 following exploration of a novel or familiar environment were qualitatively and quantitatively similar to those observed in WT mice. Furthermore, *Egr1* deficiency had no effect in CA1 on *Arc* distribution into dendrites. Since there was no detectable late-phase increase in *Arc* transcription following exploration of the novel or familiar environments in mice, it was not possible to further assess the impact of *Egr1* deficiency on *Arc* transcription in this experiment. However, since WT and *Egr1*$^{-/-}$ mice displayed similar patterns of *Arc* expression shortly after exploration, our results suggest that hippocampal neural ensembles in dentate gyrus and CA1 are normally activated during spatial exploration in *Egr1*$^{-/-}$ mice. In CA1 in particular, Guzowski et al. (1999) showed that the proportion of CA1 neurons expressing *Arc* immediately after spatial exploration in one or two environments is consistent with the proportion of pyramidal neurons that exhibit place cell properties in different environments, supporting the notion that *Arc* expression in CA1 neurons is related to the formation of a neural representation of specific environmental contexts. Hence, the normal early expression of *Arc* mRNA in CA1 pyramidal cells reported here after spatial exploration is consistent with our recent electrophysiological data showing that *Egr1*$^{-/-}$ mice can form new place cell representations normally in novel environments, although *Egr1* deficiency impairs the ability to maintain newly formed neural representations over long delays (Renaudineau et al., 2009).

CONCLUSION

This study of *Arc* mRNA expression in mice using FISH reveals several similarities, but also substantial differences in activity-dependent transcription of *Arc* in hippocampal neurons between mice and rats. We observed a single early and transient wave of *Arc* expression in the dentate gyrus and area CA1 after spatial exploration of novel or familiar environments in mice and our results show this wave of *Arc* expression was not affected in *Egr1* mutant mice, suggesting that spatial exploration recruits similar size dentate gyrus and CA1 neural ensembles in WT and *Egr1* knockout mice immediately following spatial exploration. Our results with strong neuronal activation using ECS provide evidence that the *Egr1* member of the *Egr* transcription factor family can alone control late-phases of activity-dependent hippocampal *Arc* transcription. Thus, it remains possible that a defect in the regulation of *Arc* may be one mechanism downstream of *Egr1* deficiency which could underlie some of the long-term plasticity and long-term memory deficits that have been characterized in *Egr1*$^{-/-}$ mice. In these experiments, we specifically focused on the dorsal hippocampus, a segment primarily implicated in cognitive processes of learning and memory associated with navigation and exploration (reviewed in Fanselow and Dong, 2010). Future studies might usefully extend these observations to other regions of the hippocampus and to other brain structures, in relation to forms of memory that would be associated with longer-lasting *Arc* transcription patterns in neurons of mice.

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DARPP-32, jack of all trades... master of which?

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DARPP-32 (PPP1R1B) was discovered as a substrate of cAMP-dependent protein kinase (PKA) enriched in dopamine-innervated brain areas. It is one of three related, PKA-regulated inhibitors of protein phosphatase-1 (PP1). These inhibitors seem to have appeared in early vertebrate ancestors, possibly Gnathostomes. DARPP-32 has additional important biochemical properties including inhibition of PKA when phosphorylated by Cdk5 and regulation by casein kinases 1 and 2. It is highly enriched in specific neuronal populations, especially striatal medium-size spiny neurons. As PP1 inhibitor DARPP-32 amplifies and/or mediates many actions of PKA at the plasma membrane and in the cytoplasm, with a broad spectrum of potential targets and functions. DARPP-32 also undergoes a continuous and tightly regulated cytonuclear shuttling. This trafficking is controlled by phosphorylation of Ser-97, which is necessary for nuclear export. When phosphorylated on Thr-34 and dephosphorylated on Ser-97, DARPP-32 can inhibit PP1 in the nucleus and modulate signaling pathways involved in the regulation of chromatin response. Recent work with multiple transgenic and knockout mutant mice has allowed the dissection of DARPP-32 function in striato-nigral and striato-pallidal neurons. It is implicated in the action of therapeutic and abused psychoactive drugs, in prefrontal cortex function, and in sexual behavior. However, the contribution of DARPP-32 in human behavior remains poorly understood. Post-mortem studies in humans suggest possible alterations of DARPP-32 levels in schizophrenia and bipolar disorder. Genetic studies have revealed a polymorphism with possible association with psychological and psychopathological traits. In addition, a short isoform of DARPP-32, t-DARPP, plays a role in cancer, indicating additional signaling properties. Thus, DARPP-32 is a non-essential but tightly regulated signaling hub molecule which may improve the general performance of the neuronal circuits in which it is expressed.

Keywords: dopamine, glutamate, cAMP, protein-phosphatase 1, CDK5, behavior, addiction, Parkinson's disease

What we know today about signal transduction derives from early studies of the glycogen metabolism. Sutherland (1972) identified cAMP as the intracellular second messenger which mediates the action of glucagon and epinephrine on glycogen degradation through activation of glycogen phosphorylase. Edwin G. Krebs and Edmond H. Fischer identified the role of the first protein kinase (PKA), phosphorylase kinase, in activating phosphorylase (see Fischer, 1997). In 1968, Krebs and Walsh discovered that cAMP action was mediated by activation of cAMP-dependent PKA, which activated phosphorylase kinase (Walsh et al., 1968). Paul Greengard and his collaborators then showed that similar cascades of biochemical reactions were critical in the action of neurotransmitters in the nervous system (Nestler and Greengard, 1983; Greengard, 2001). They identified the first dopamine (DA) receptor by its positive coupling to adenylyl-cyclase (Kebabian et al., 1972) and downstream protein phosphorylation (Krueger et al., 1975). To better characterize the mode of action of neurotransmitters in various brain regions, Walaas et al. (1983b) undertook a systematic regional study of proteins phosphorylated by second messengers-activated protein kinases. Some of these substrates, or "third messengers" were evenly distributed whereas others were highly enriched in specific brain regions. For example, Walaas observed the enrichment of a 32-kDa soluble PKA substrate in striatal slices extracts. The phosphorylation of this protein highly expressed in DA-innervated brain regions, was regulated by DA and cAMP and it was termed DARPP-32 (dopamine and cAMP-regulated phospho-protein Mr 32,000; Walaas et al., 1983a). DARPP-32 had properties similar to those of protein phosphatase-1 (PP1) inhibitor-1 and was shown to be a potent inhibitor of PP1 when phosphorylated by PKA (Hemmings et al., 1984a). Hence, DARPP-32 is also known as phospho-protein phosphatase-1 regulatory subunit 1B (PPP1R1B), although its properties are not restricted to PP1 inhibition (see below). DARPP-32 is expressed only in some specific neuronal populations in the brain and at low levels in a few non-neuronal cell types in the brain (e.g., tanycytes, choroid plexus) and other organs (e.g., adrenal medulla, parathyroid cells, kidney thick ascending limb of the loop of Henle; Ouimet et al., 1984; Hemmings and Greengard, 1986; Meister et al., 1989). Not all of these cells express adenylyl-cyclase-coupled DA receptors (D1R and D5R) and DARPP-32 phosphorylation on

the cAMP site (Thr-34; Hemmings et al., 1984c) can be regulated by many other extracellular messengers (see Greengard et al., 1999; Girault and Greengard, 2004; Svenningsson et al., 2004 for reviews). Thus, DARPP-32 has been implicated in many physiological and pathological responses involving the basal ganglia, and other brain regions and peripheral organs. Here we review the major molecular properties of DARPP-32 and we focus on recent findings on its possible roles based on studies in animal models as well as on correlations in humans. We also try to put its molecular properties in perspective with the known function of striatal neurons.

MOLECULAR PROPERTIES OF DARPP-32

DARPP-32 IS A PHOSPHORYLATION-DEPENDENT INHIBITOR OF PP1 REGULATED BY CYCLIC NUCLEOTIDES-DEPENDENT PROTEIN KINASES

The biochemical properties of DARPP-32 have raised a great interest because of the surprising complexity of the effects of its phosphorylation at various sites. The most extensively studied is the phosphorylation of Thr-34 which turns DARPP-32 into a potent inhibitor of PP1 (Hemmings et al., 1984a). Thr-34 in DARPP-32, like Thr-35 in inhibitor-1 are excellent substrates for both PKA and cGMP-dependent protein kinases (Hemmings et al., 1984b). They are dephosphorylated by the calcium/calmodulin activated phosphatase 2B (PP2B, also known as calcineurin or PPP3) and by the catalytic subunit of protein phosphatase 2A (PP2A or PPP2) *in vitro* (Hemmings et al., 1984a; King et al., 1984) and in striatal neurons (Halpain et al., 1990; Nishi et al., 1999).

Protein phosphatase-1 is a major, highly conserved Ser/Thr phosphatase, involved in virtually all cell functions including cell cycle, muscle contraction, carbohydrate metabolism, neuronal signaling, and transcription (see Bollen et al., 2010 for a recent review). The activity of PP1 catalytic subunit (PP1c) is controlled by numerous interacting proteins acting as targeting subunits, substrates, and/or inhibitors (Bollen et al., 2010). Most of these proteins contain a degenerate docking sequence K/R-K/R-V/I-x-F/W necessary for binding PP1c, without changing its conformation (Egloff et al., 1997; Bollen et al., 2010). It has been shown by X-ray crystallography that this motif interacts with a hydrophobic channel located on the opposite side of PP1c active site (Egloff et al., 1997). However, binding to this motif does not inhibit PP1, implying that other domains of inhibitors must interact with the phosphatase to regulate its activity. The docking motif is also found in targeting subunits and can compartmentalize PP1, bringing it into proximity to its substrates, including GADD34, the myosin phosphatase targeting subunit MYPT1, PNUTS, and spinophilin (see Bollen et al., 2010 for references). Among PP1 inhibitors the PPP1R1 family includes inhibitor-1 (PPP1R1A; Aitken et al., 1982), DARPP-32 (PPPR1B), and the much less well characterized inhibitor of protein phosphatase 5 (IPP-5 or PPP1R1C; Wang et al., 2008), which share a conserved region of about 50-residues (**Figure 1**). Phylogenetic studies using immunoblotting detected DARPP-32-like proteins in dopaminoceptive brain regions from representative members of the amniote vertebrate classes (birds and reptiles), while none was identified in dopaminoceptive brain regions from representative members of the anamniote vertebrate classes (bony fishes and amphibians; Hemmings and Greengard, 1986). With the same approach inhibitor-1-like immunoreactivity was detected in anamniote and amphibian representatives (Hemmings et al., 1992). However such studies are limited by the lack of conservation of epitopes. Indeed sequence comparisons show that all three PPP1R1 family members are actually found in Osteichthyes including bony fish, amphibians lizards, birds, and mammals (**Figure 2**). We also detected short predicted sequences similar to PPP1R1B and PPP1R1C in Elephant shark genome (http://esharkgenome.imcb.a-star.edu.sg/), a cartilaginous fish, although the sequence coverage is not yet sufficient to determine precisely the conservation of these genes in Chondrichthyes. In contrast no homologous sequence was found in Petromyzontoids (jawless fish, e.g., lamprey) and invertebrates. These data, albeit still incomplete, suggest that the PP1R1 family arose in early vertebrate ancestors, possibly in Gnathostomes, which encompass both bony and cartilaginous fish.

pT34-DARPP-32 inhibits PP1c with mixed competitive and uncompetitive kinetics (Hemmings et al., 1984a, 1990). Residues 8-38, which are highly conserved between the three members of the PPP1R1 family are necessary and sufficient for PP1 inhibition (Hemmings et al., 1990). The N-terminal region of DARPP-32 encompasses a K_7KIQF motif that corresponds to the canonical PP1 interacting motif. This motif is essential for PP1 inhibition (Kwon et al., 1997) and probably accounts in part for the ability of dephospho-DARPP-32 or Thr-34-Ala DARPP-32 to inhibit

FIGURE 1 | Multiple sequence alignment of human PPP1R1 family members. Human amino acid sequences of PPP1R1C (IPP-5, gi|17389867|gb|AAH17943.1|), PPP1R1A (I-1, gi|48146123|emb|CAG33284.1|), and PPP1R1B (DARPP-32, gi|119580986|gb|EAW60582.1|) were obtained from NCBI data base and aligned by Clustalview. Identical residues are shaded black and similar ones gray. The known phosphorylation sites are in red and the PP1 binding motif in green.

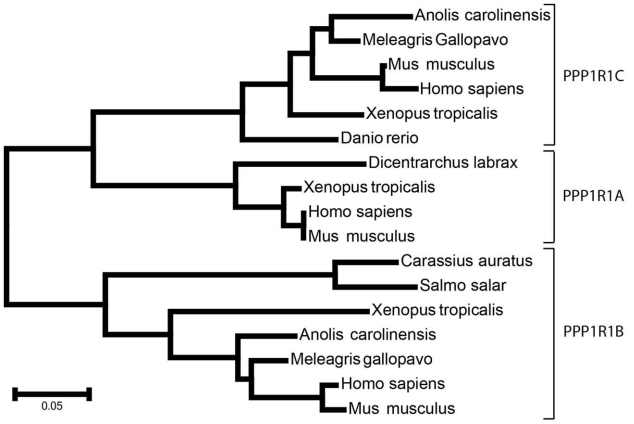

FIGURE 2 | Phylogeny of PPP1R1 family. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch lengths = 1.395 is shown. The tree is drawn to scale, with branch lengths in the same units as the evolutionary distances used to infer the phylogenetic tree (number of amino acid differences per site). The evolutionary distances were computed using the p-distance method (Nei and Kumar, 2000). Positions containing gaps and missing data were eliminated. There were a total of 42 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al., 2011). The 17-amino acid sequences analyzed were: PPP1R1A gi|326935910| ref|XP_003214007.1| (*Meleagris gallopavo*, wild turkey), gi|227337247|gb|ACP21475.1| (*Carassius auratus*, gold fish), gi|48146123| emb|CAG33284.1| (*Homo sapiens*), gi|10946734| ref|NP_067366.1| (*Mus musculus* house mouse), gi|62859473| ref|NP_001015900.1| (*Xenopus tropicalis*, Western clawed frog); PPP1R1B: gi|119580986| gb|EAW60582.1| (*Homo sapiens*), gi|21536256| ref|NP_659077.1| (*M. musculus*), gi|156717604| ref|NP_001096342.1| (*X. tropicalis*), gi|327275684| ref|XP_003222603.1| (*Anolis carolinensis*, Carolina anole, a lizard), gi|326936204| ref|XP_003214147.1| (*M. gallopavo*), gi|213515022| ref|NP_001133145.1| (*Salmo salar*, Atlantic salmon); PPP1R1C: gi|50540138| ref|NP_001002538.1| (*Danio rerio*, zebrafish), gi|327278420| ref|XP_003223960.1| (*A. carolinensis*), gi|122889495| emb|CAM14502.1| (*M. musculus*), gi|17389867| gb|AAH17943.1| (*Homo sapiens*), gi|284413766| ref|NP_001165134.1| (*X. tropicalis*), gi|326922651| ref|XP_003207562.1| (*M. gallopavo*).

PP1 with an IC50 in the micromolar range (Desdouits et al., 1995a). Phosphorylation of Thr-34 provides a high affinity inhibition (nanomolar IC50), whereas the replacement of pThr by pSer or Glu is much less efficient (Hemmings et al., 1990; Desdouits et al., 1998). The two Pro residues flanking Thr-34 are also important, as well as the distance between the KKIQF and PpTP motifs (Huang et al., 1999). Thus, it is highly likely that inhibition by DARPP-32 results from a bidentate interaction with PP1c, involving regions located on two opposite sides of the phosphatase: binding of the KKIQF motif to the superficial hydrophobic channel and interaction of PpTP with the active site in a conformation that does not allow dephosphorylation (Hemmings et al., 1990; Kwon et al., 1997; Huang et al., 1999). Like several other PP1 inhibitors, DARPP-32 appears to be an intrinsically disordered protein with little identifiable secondary structure (Neyroz et al., 1993; Dancheck et al., 2008; Marsh et al., 2010), a particularity that may be well suited for a flexible interaction with two diametrically opposed binding sites on PP1c.

DARPP-32 IS PHOSPHORYLATED BY SEVERAL KINASES WITH MULTIPLE CONSEQUENCES

DARPP-32 is regulated by multiple phosphorylation sites (**Figure 3**). Cdk5, a cyclin-dependent kinase family member activated in neurons by its co-factor p35, phosphorylates DARPP-32 on Thr-75 (Bibb et al., 1999). When it is phosphorylated on Thr-75, DARPP-32 inhibits PKA *in vitro* with a purely competitive kinetics and a Ki of 2.7 μM using cAMP-regulated phospho-protein of 21 kDa (ARPP-21, also known as regulator of calmodulin signaling, RCS) as a substrate (Bibb et al., 1999). DARPP-32 is also phosphorylated on Ser-45 and Ser-97 (Ser-102 in rat sequence) by casein kinase 2 (CK2; Girault et al., 1989a). Phosphorylation by CK2 increases the action of PKA on Thr-34, but not that of cGMP-dependent protein kinase on that site (Girault et al., 1989a). It is not known whether this effect results from phosphorylation of Ser-45 or Ser-97, or both, nor what the physiological relevance of this facilitation is *in vivo*. An important effect of phosphorylation of Ser-97 is to enhance the nuclear export of DARPP-32 (Stipanovich et al., 2008 see below). DARPP-32 is also phosphorylated on Ser-130 (Ser-137 in rat) by casein kinase 1 (CK1; Desdouits et al., 1995b), and this phosphorylation decreases the dephosphorylation of Thr-34 by calcineurin, *in vitro* and *in vivo* (Desdouits et al., 1995c). The molecular mechanisms underlying the modulatory effects of CK1 and CK2 are not elucidated. Since DARPP-32 appears to be intrinsically unstructured, it is unlikely that the facilitation of PKA action and inhibition of calcineurin action result from allosteric effects *stricto sensu*. Instead they may be related to changes in interactions with the relevant enzymes through distant binding sites, and/or to intramolecular folding and interactions within the DARPP-32 molecule, as suggested by NMR studies (Dancheck et al., 2008). In addition to the sites described above, Ser-192 has also been found to be phosphorylated in mouse brain (Jin et al., 2005), but this site is not conserved in several mammalian species and its functional role, if any, is not known.

The dephosphorylation of DARPP-32 by various protein phosphatases has been studied *in vitro* and in intact cells. Calcineurin dephosphorylates Thr-34 (King et al., 1984; Halpain et al., 1990; Nishi et al., 1999), PP2A dephosphorylates Thr-34, Ser-45, Thr-75, and Ser-97 (Girault et al., 1989a; Hemmings et al., 1990; Bibb et al., 1999; Nishi et al., 1999). In striatal neurons dephosphorylation of Ser-130 is mostly accounted for by PP2C (Desdouits et al., 1998). PP2A is an heterotrimeric enzyme comprising a catalytic subunit C, a scaffolding subunit A, and a variable regulatory subunit B (Janssens et al., 2008). The B56δ subunit (also known as PR61δ, B′δ or PPP2R5D) is enriched in the striatum and mediates dephosphorylation of Thr-75 and Ser-97 in response to PKA activation (Nishi et al., 2000a; Ahn et al., 2007a; Stipanovich et al., 2008). The PR72/B′ (PPP2R3A) subunit, which contains two Ca^{2+} binding sites, accounts for the Ca^{2+}-induced dephosphorylation of Thr-75 (Nishi et al., 2002; Ahn et al., 2007b). Thus, the activity of PP2A appears to be regulated by multiple pathways which depend on the nature of the regulatory and inhibitory subunits that are expressed in the cell. An additional layer of regulation potentially important in striatal neurons has been revealed recently by the discovery that proteins related to ARPP-16, which is enriched in striatal neurons (Girault et al., 1990), are potent inhibitors of PP2A (Gharbi-Ayachi et al., 2010; Mochida et al., 2010). This effect requires

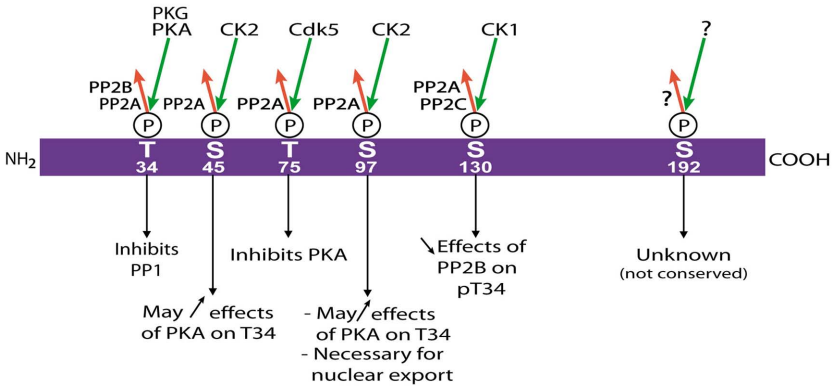

FIGURE 3 | Multisite phosphorylation of DARPP-32. Schematic representation of mouse DARPP-32 with the residues that have been found phosphorylated *in vivo* (Thr-34, Ser-45, Thr-75, Ser-97, Ser-130, Ser-192). The identified kinases are indicated above green arrows (phosphorylation), and protein phosphatases beside the red arrows (dephosphorylation). The role of the phosphorylation is given for each residue. See the text for discussion and references. P, phosphate.

phosphorylation by Greatwall, a PKA that has several orthologous isoforms in mammals (microtubule associated serine/threonine kinase like, MASTL1-4). The role of this pathway on PP2A regulation and indirectly on PP1 in the striatum remains to be investigated. Another interesting regulation of PP2A in D2R-expressing neurons is related to its recruitment by β-arrestin following receptor internalization (Beaulieu et al., 2005), but its role in the regulation of DARPP-32 has not been investigated.

The phosphorylation sites of DARPP-32 and their effects on kinases and phosphatases, including those which act on DARPP-32 itself, weave a complex network of positive and negative feedback and feedforward loops. Several computational models have been built to better understand the complex regulation of these four sites and predict the output of the pathway depending on the levels of DA and glutamate, or of cAMP and Ca^{2+} (Fernandez et al., 2006; Lindskog et al., 2006; Barbano et al., 2007; Le Novère et al., 2008; Nakano et al., 2010; Qi et al., 2010). Kinetic models using differential equations showed that DARPP-32 is not only a robust signal integrator, but that its response also depends on the delay between cAMP and calcium signals (Fernandez et al., 2006) and that Thr-34 phosphorylation is potentiated by the coincidence of the two stimuli (Lindskog et al., 2006). DARPP-32 appears capable to translate the various states of glutamate and DA input into distinct steady states and/or dynamic responses (Qi et al., 2010) and the positive feedback loop consisting of PKA, PP2A, and Thr-75 phosphorylation may serve as a major switch for inducing LTD and LTP (Nakano et al., 2010). Interestingly this loop displayed robust bi-stable responses but was disrupted by high basal levels of DA. Other authors have used large-scale numerical simulation and sophisticated mathematical approach to model the DARPP-32 network and evaluate its resistance to perturbations (Barbano et al., 2007)[1]. They showed that the global network topology stabilized the net state of DARPP-32 phosphorylation in response to variations of the input levels of DA and glutamate, despite significant perturbation to the concentrations and levels of activity of a number of intermediate chemical species (Barbano et al., 2007). Interestingly the whole network used in the model was necessary to provide this resistance to perturbation, providing a possible *a posteriori* justification for its apparent complexity. Altogether these modeling studies indicate that DARPP-32 is a very robust sensor of glutamate and DA inputs with intrinsic abilities to stably orient the cell responses in the presence of noise. This may suggest that a major function of DARPP-32 is to increase the reliability of signal processing in medium-size spiny neurons (MSNs) of the striatum.

DARPP-32 IS A MESSENGER BETWEEN THE CYTOPLASM AND THE NUCLEUS

Since PP1 dephosphorylates many targets of PKA in neurons, DARPP-32 has been implicated in the regulation by DA of several ion channels in striatal neurons, including the AMPA and NMDA glutamate receptors (NMDAR) and voltage-gated Na^+ and Ca^{2+} channels (see Svenningsson et al., 2004 for a review). A recent study has revealed an additional level of complexity concerning the signaling properties of DARPP-32, by showing its regulated cytonuclear trafficking (Stipanovich et al., 2008). Although the presence of DARPP-32 immunoreactivity in the nucleus of some striatal MSNs, but not in others, was clearly established by electron microscopy early in the study of this protein (Ouimet and Greengard, 1990), the significance of this observation was unclear. Studies in neurons in culture with the inhibitor of nuclear export leptomycin B revealed that DARPP-32 undergoes a continuous shuttling between the cytoplasm and the nucleus (Stipanovich et al., 2008). Its import is facilitated by several sequences in the N-terminal region, while the nuclear export requires a specific sequence with nuclear export signal features and the phosphorylation of Ser-97. Accordingly, the protein is mostly found in

[1]The approach of Barbano et al. (2007) does not require knowledge of initial concentrations or kinetic parameters, but uses random arbitrary values to calculate steady state equilibrium concentrations of all chemical reagents for various ratios of DA and glutamate inputs. Simulations are repeated in the presence of superimposed noise. The results of each simulation represent coordinates of a single point in a space with as many dimensions as reagents in the network. The authors then use non-linear dimensionality reduction methods, such as locally linear embedding, to compare the resulting curves in the absence or presence of various amounts of noise and determine the resistance of the network to perturbations.

the nucleus in knock-in mice bearing a Ser-97-Ala DARPP-32 mutation (Stipanovich et al., 2008). Treatment of mice with addictive drugs or operant learning increases the proportion of nuclear DARPP-32, a response mimicked *in vitro* by cAMP analogs (Stipanovich et al., 2008). Dephosphorylation of Ser-97 by PP2A is an important factor in these regulations. It can be triggered by activation of the isoform containing the B56δ subunit (Stipanovich et al., 2008), as well as by other signaling pathways (Matamales and Girault, unpublished observations).

PP1 is present in the nucleus of cells where it plays important roles (Moorhead et al., 2007) with PP1α being the predominant nuclear isoform in MSNs (Ouimet et al., 1995). Following activation of D1R, pT34-DARPP-32 is enriched in the nucleus and this facilitates the phosphorylation of histone H3 on Ser-10 (Stipanovich et al., 2008), a site dephosphorylated by PP1 (Hsu et al., 2000). Phosphorylation of histone H3 Ser-10 during the interphase promotes detachment of specific regions from the heterochromatic scaffold to allow decondensation and gene expression, in combination with other histone post-translational modifications (Johansen and Johansen, 2006). Since PP1 has many substrates in the nucleus (Moorhead et al., 2007), it is likely that the consequences of the accumulation of pT34-DARPP-32 has multiple consequences on chromatin and other nuclear targets. For example, a recent report shows that DARPP-32 interacts with tra2-beta1, a factor involved in the regulation of alternative splicing (Benderska et al., 2010). This latter observation suggests a possible role of DARPP-32 in the regulation of alternative splicing an important but little understood process in neurons.

DISTINCT ROLES OF DARPP-32 IN STRIATO-NIGRAL AND STRIATO-PALLIDAL MSNs

DARPP-32 IS HIGHLY CONCENTRATED IN ALL STRIATAL MEDIUM-SIZE SPINY NEURONS

DARPP-32 concentrations are the highest (20–50 μM) in striatal MSNs (Hemmings and Greengard, 1986). The striatum, which consists of a dorsal region, the caudate–putamen (CP), and a ventral region, the nucleus accumbens (NAc), is the major entry station of the basal ganglia. It is comprised of MSNs which are GABAergic efferent neurons making up to 95% of striatal neurons in rodents, and of several types of interneurons, including large cholinergic and diverse medium-size GABAergic neurons (Tepper and Bolam, 2004). MSNs receive massive glutamatergic inputs from virtually all cortical areas and the thalamus, and a dense dopaminergic innervation from the *substantia nigra pars compacta* (dorsolateral CP) and the ventral tegmental area (VTA; dorsomedial CP and NAc). MSNs belong to two different populations which project to the *substantia nigra pars reticulata* either directly or indirectly through relays in the lateral globus pallidus and subthalamic nucleus. D1 DA receptors (D1R) are preferentially expressed in striato-nigral MSNs of the direct pathway and D2R in striato-pallidal MSNs of the indirect pathway (Gerfen et al., 1990; see also Valjent et al., 2009; Bertran-Gonzalez et al., 2010 for recent reviews). Thus, the circuits in which MSNs play an essential role integrate signals coming from many brain regions with modulatory information coded by DA neurons in relation with reward prediction errors (Schultz, 2002) and motivational salience (Bromberg-Martin et al., 2010). The basal ganglia loops control many aspects of movements and motivation and their function has been proposed to be related to action selection (Mink, 1996; Redgrave et al., 1999). This is dependent on the balance between the two trans-striatal circuits, involving the direct and indirect pathways. Their disequilibrium accounts for the consequences of the striatal alterations in several pathological conditions, including Parkinson's disease due to the lack of DA following degeneration of DA neurons and addiction, related to the common capacity of abused substances to increase extracellular DA (Di Chiara, 1999). DARPP-32 has been implicated in various aspects of striatal functions and dysfunctions, with distinct consequences in the direct and indirect pathways.

DARPP-32 IS DIFFERENTIALLY REGULATED IN THE TWO POPULATIONS OF MSNs

DARPP-32 is present in virtually all MSNs (Ouimet et al., 1998) and equally expressed in the D1R-expressing striato-nigral neurons (indirect pathway) and D2R-expressing striato-pallidal neurons (direct pathway; Bertran-Gonzalez et al., 2008). A number of drugs have been reported to alter DARPP-32 phosphorylation at its various sites in striatal slices *in vitro* and in the striatum *in vivo* (see Svenningsson et al., 2004). Some of these studies apparently had contradictory results, showing for example that agents that either increase DA transmission, such as cocaine, or block DA transmission, such as antipsychotics, both increased DARPP-32 phosphorylation on Thr-34 (Svenningsson et al., 2000). The fact that increased phosphorylation was due to stimulation of D1R or blockade of D2R, and that the latter effect was prevented by A2a antagonists[2] suggested that this effect was due to the different regulation of DARPP-32 in D1R- and D2R-expressing MSNs (Svenningsson et al., 2000). This hypothesis was formally proven by the use of bacterial artificial chromosome (BAC) transgenic mice expressing DARPP-32 fused to two different peptide tags, under the control of *drd1a* (D1R) or *drd2* (D2R) promoters, respectively (Bateup et al., 2008). Selective immunoprecipitation with antibodies against one peptide tag or the other showed that, as expected, cocaine-induced phosphorylation of DARPP-32 at Thr-34 in D1Rneurons and decreased it in D2R- neurons, while it decreased Thr-75 phosphorylation in D1R- and increased it in D2R-neurons (Bateup et al., 2008). Haloperidol increased selectively Thr-34 phosphorylation in D2R-neurons. Surprisingly however, the *in vivo* effects of selective D1R or D2R agonists appeared more complex: SKF81297, a D1R agonist, increased Thr-34 phosphorylation in both neuronal populations, whereas quinpirole, a D2R agonist, decreased Thr-34 and increased Thr-75 phosphorylation in both neuronal populations (Bateup et al., 2008). The effects of the D2 agonist on D1R-expressing neurons were easily explained by the decreased release of endogenous DA following the stimulation of D2 autoreceptors on DA terminals. In contrast, the mechanisms of the effects of the D1 agonist on D2R-expressing neurons were not completely clear and may reflect the recruitment of extrastriatal D1R. It is interesting to note that in the study of Bateup et al. (2008), as in others (Bertran-Gonzalez

[2]A2a receptors are selectively expressed in striato-pallidal neurons in which they stimulate adenylyl-cyclase, thereby opposing the effects of D2R which inhibit adenylyl-cyclase.

et al., 2008), the *in vivo* actions of cocaine that non-selectively increases extracellular DA and other monoamines, appeared more specific on striato-nigral D1R-expressing neurons than those of selective D1 agonists. This unexpected observation suggests that the coordinated stimulation of D1R and D2R by endogenous DA is particularly well suited to exert contrasted and opposing effects on the two populations of MSNs, probably reflecting a fundamental intrinsic property of the functional organization of the basal ganglia.

THE FUNCTIONAL CONSEQUENCES OF THE ABSENCE OF DARPP-32 ARE DISTINCT IN THE TWO POPULATIONS OF MSNs

To investigate the role of DARPP-32 in the two populations of striatal MSNs from a behavioral standpoint, Bateup et al. (2010) have used conditional knockout in D1R or in D2R-expressing MSNs. The absence of DARPP-32 in D1R MSNs reduced basal and cocaine-induced locomotor activity whereas the loss of DARPP-32 in D2R MSNs had the opposite effect. The opposite effects on motor behavior in the two populations of striatal neurons may explain why the complete knockout had relatively little effect on spontaneous motricity (Fienberg et al., 1998; Hiroi et al., 1999).

In a rodent model of Parkinson's disease, in which the striatal dopaminergic input is destroyed by 6-OH-DA, chronic administration of L-DOPA-induced phosphorylation of DARPP-32 at Thr-34 (Santini et al., 2007). Chronic L-DOPA treatment of DA-lesioned rodents often triggers abnormal involuntary movements (AIMs), which are thought to model L-DOPA-induced dyskinesia observed in patients (Cenci, 2007). Interestingly, phosphorylation of both DARPP-32 and extracellular signal regulated kinase (ERK) was correlated with the occurrence of AIMs (Santini et al., 2007). D1R is critical for the development of L-DOPA-induced AIMS (Darmopil et al., 2009) and L-DOPA selectively induced ERK phosphorylation in D1R-expressing neurons (Santini et al., 2009). AIMs induction was diminished when L-DOPA was administered with a drug preventing ERK activation or in DARPP-32 knockout mice (Santini et al., 2007). Although this suggests a causal relationship between DARPP-32 phosphorylation and ERK activation (Santini et al., 2007), other authors found that ERK phosphorylation was not blocked in the dorsal striatum in similar conditions (Gerfen et al., 2008). The reason for this discrepancy is not known. However, the prominent role of DARPP-32 in striato-nigral neurons in the generation of L-DOPA-induced AIMs was shown by their absence in mice in which DARPP-32 was deleted in D1R-expressing neurons, but not in those in which the deletion was induced in D2R-expressing neurons (Bateup et al., 2010). This result is in agreement with those obtained in D1R knockout mice (Darmopil et al., 2009). In conclusion, the ERK pathway and DARPP-32 in striato-nigral neurons appear to be both involved in the appearance of AIMs but the precise relationship between the two is not fully understood.

MULTIPLE ROLES OF DARPP-32 IN REWARD AND THE ACTION OF DRUGS OF ABUSE

It is well established that the mesencephalic DA neurons play an important role in reward-related behaviors (see for example Schultz, 2002 for a review). Little is known about the involvement of DARPP-32 in physiological reward-controlled learning. Although DARPP-32 seems not to be crucial for reward learning, the reversal learning after a conditioning operant task by nose poking was impaired in DARPP-32 KO mice (Heyser et al., 2000). In knock-in mice with a Ser-97-Ala point mutation, operant learning was normal but the motivation for food reward was decreased (Stipanovich et al., 2008). Drugs of abuse share the ability to increase DA transmission (Wise, 1987; Di Chiara, 1999), and the role of DARPP-32 in mediating their actions has been studied for molecules as different as cocaine, amphetamine, nicotine, caffeine, ethanol, and opiates (see Nairn et al., 2004 for a review). One of the signaling pathways which appear to be important for the long-term behavioral effects of drugs of abuse is the ERK cascade which is activated in response to all tested drugs of abuse (Valjent et al., 2000, 2004; Ibba et al., 2009). ERK phosphorylation requires activation of both D1R and NMDAR, occurs in D1R-expressing neurons, and is prevented in DARPP-32 knockout or Thr-34-Ala knock-in mice (Valjent et al., 2005). Thus, although DARPP-32 is not necessary for the potentiation of NMDAR by D1R that leads to ERK phosphorylation in neurons in culture (Pascoli et al., 2011), it appears to be necessary for the activation of ERK by various drugs of abuse *in vivo* (Valjent et al., 2005). DARPP-32 regulates dephosphorylation of ERK by controlling the state of phosphorylation and activity of the non-receptor striatal-enriched phosphatase (STEP) and also acts upstream in the pathway (Valjent et al., 2005). Here we summarize results implicating DARPP-32 in the actions of drugs of abuse and focus on recent work, including studies in which the role of individual phosphorylation sites of DARPP-32 has been studied (Svenningsson et al., 2003).

COCAINE AND AMPHETAMINE

Cocaine inhibits reuptake of dopamine and amphetamine triggers the release of dopamine from nerves terminals. Acute exposure to cocaine or amphetamine increased the phosphorylation of DARPP-32 at Thr-34 and decreased the phosphorylation at Thr-75 in striatal slices (Nishi et al., 2000b). *In vivo*, an acute injection of cocaine (20 mg/kg) increased Thr-34 phosphorylation in D1R-expressing neurons (Bateup et al., 2008). This treatment had opposite effects on Thr-75 phosphorylation in D1R striato-nigral neurons (decreased phosphorylation) and in D2R striato-pallidal neurons (increased phosphorylation), with an overall decreased phosphorylation (Bateup et al., 2008). In contrast, chronic exposure to cocaine-induced an increase of phosphorylation at Thr-75 (Bibb et al., 2001). This effect was attributed to the up-regulation of Cdk5, resulting from an over-expression of Δ-Fos B after a chronic exposure (Bibb et al., 2001).

Repeated administration of psychostimulants in rodents is accompanied by an increased locomotor response, termed locomotor or psychomotor sensitization (Vezina and Leyton, 2009). Locomotor sensitization was increased when cocaine was repeatedly administered in the presence of a Cdk5 inhibitor (Bibb et al., 2001), supporting a negative role of the phosphorylation of Thr-75 and/or other targets. However, contrary to what might have been predicted, Thr-34-Ala point mutation increased locomotor sensitization to repeated administration of cocaine in knock-in mutant mice, whereas Thr-75-Ala mutation point prevented sensitization (Zachariou et al., 2006). An increased sensitization was also observed in complete knockout mice in response to repeated

cocaine (Hiroi et al., 1999). A strong and long-lasting sensitization can be induced in rats or mice in response to a single injection of cocaine or amphetamine, attesting to the powerful effects of these drugs on neuronal plasticity (Vanderschuren et al., 1999; Valjent et al., 2010). Using this protocol, sensitization to a single injection of cocaine was markedly decreased in Thr-34-Ala mutant mice (Valjent et al., 2005). These discrepancies may be related to different mechanisms underlying sensitization in response to single and repeated drug injections and/or to minor differences in the experimental protocols which are highly sensitive to conditioned cues (Vezina and Leyton, 2009).

Conditioned place preference (CPP) to cocaine was diminished in full knockout (Hiroi et al., 1999) and in Thr-34-Ala knock-in DARPP-32 mutant mice (Zachariou et al., 2002). Locomotor sensitization and CPP were also decreased in DARPP-32 Ser-97-Ala mutant mice, in which DARPP-32 is mostly trapped in the nucleus (Stipanovich et al., 2008). Acquisition of stable cocaine self-administration required significantly more time in Thr-34-Ala mice, although both Thr-34-Ala and Ser-130-Ala DARPP-32 mutant mice self-administered more cocaine than their respective wild-type controls (Zhang et al., 2006). Thus it appears that DARPP-32 is involved in the chronic effects of psychostimulants, although its role is not simple. Its loss of function delays or impairs the acquisition of cocaine conditioned behavior, but may limit locomotor sensitization to repeated administration and self-administration.

MORPHINE

Morphine and heroin are thought to exert their addictive effects at several levels. They increase the firing of DA neurons by decreasing at the presynaptic level the inhibitory input to these neurons and also act directly in the NAc (see Luscher and Malenka, 2011 for a review). Acute locomotor effects of morphine were decreased in Thr-34-Ala mutant mice, whereas locomotor sensitization to repeated injections and CPP were unaltered (Borgkvist et al., 2007). In contrast, sensitization to a single injection of morphine was markedly decreased in Thr-34-Ala mutant mice (Valjent et al., 2010), and in Ser-97-Ala mutant mice (Stipanovich et al., 2008). Thus, modulation of the behavioral effects of psychostimulants and morphine by DARPP-32 appear to be different depending on whether the drug is administered once or repeatedly. Following a single administration DARPP-32 seems to amplify the effects of the drugs and to be necessary for long-term plasticity. In contrast, following repeated administration, it appears to moderate the effects in a somewhat homeostatic fashion.

CAFFEINE

Caffeine is a commonly used minor psychostimulant, which exerts its effects mostly through antagonism of A2 adenosine receptors ($A_{2A}R$), located in the striato-pallidal neurons of the indirect pathway (Ledent et al., 1997). It is not considered as a drug of abuse and has little or no addictive properties (Satel, 2006). $A_{2A}R$ are coupled to G_{olf} and stimulate cAMP production (Kull et al., 2000; Corvol et al., 2001). DARPP-32 is necessary to mediate the psychostimulant effects of caffeine (Lindskog et al., 2002). This may be related to the increase in Thr-75 phosphorylation, which results from an inhibition of PP2A (Lindskog et al., 2002) and occurs selectively in striato-pallidal neurons (Bateup et al., 2010). These results further support the precise functional balance between the striato-nigral and striato-pallidal neurons and suggest that decreased signaling in one pathway (here PKA inhibition through phospho-Thr-75-DARPP-32) has effects similar to stimulation of signaling in the other pathway (e.g., stimulation of PKA/phospho-Thr-34-DARPP-32 by D1R).

NICOTINE

Nicotine enhances dopamine release by the nigrostriatal and preferentially mesolimbic dopaminergic systems, *in vitro* and *in vivo* (Giorguieff Chesselet et al., 1979; Imperato et al., 1986). In striatal slices nicotine at low concentration (1 μM) decreased phosphorylation of DARPP-32 at Thr-34, whereas at high concentration (100 μM) it increased phosphorylation of Thr-34, as well as Ser-102, Ser-137, and, with a delay, decreased Thr-75 phosphorylation (Hamada et al., 2004, 2005). *In vivo*, however, nicotine increased phosphorylation of both Thr-34 and Thr-75 (Zhu et al., 2005). Interestingly DARPP-32 knockout mice had an increased responsiveness to nicotine including a higher oral intake of the drug, suggesting that the "inhibitory" effect of Thr-75 plays a predominant role (Zhu et al., 2005).

THE ROLE OF SEROTONIN AND NOREPINEPHRINE

Psychostimulants increase not only extracellular dopamine but also serotonin (5OH-tryptamine, 5HT) and norepinephrine. In the striatum, stimulation of 5HT-4 and 5HT-6 receptors increases phosphorylation of DARPP-32 on Thr-34 and decreases its phosphorylation on Thr-75, while stimulation of 5HT-2 receptors enhances Ser-130 (Ser-137 in the rat) phosphorylation (Svenningsson et al., 2002a). Similar effects were observed after acute or chronic administration of the selective serotonin reuptake inhibitor fluoxetine (Svenningsson et al., 2002b). The regulation of DARPP-32 by 5HT may be functionally relevant since the behavioral effects of various agents that raise 5HT levels *in vivo* including 5-OH-tryptophan, *p*-chloroamphetamine, and fluoxetine were decreased in DARPP-32 knockout mice (Svenningsson et al., 2002a,b). The phosphorylation of DARPP-32 on the sites mentioned above was also regulated following administration of psychotomimetic compounds acting through diverse mechanisms, including amphetamine, lysergic acid diethylamide (LSD, predominantly a 5HT2A agonist), and phencyclidine (an NMDA antagonist; Svenningsson et al., 2003). The behavioral effects of these drugs were altered in knock-in mice with point mutations of either Thr-34 or Ser-130 (Svenningsson et al., 2003). Interestingly, DARPP-32 Thr-34 phosphorylation is also regulated by adrenoceptors in striatal slices with a stimulatory effect of β1-adrenoceptors in both striato-nigral and striato-pallidal neurons (Hara et al., 2010). These results support the implication of DARPP-32 far beyond the DA receptors that may contribute to the role of this protein in the action of drugs of abuse.

ROLE OF DARPP-32 IN OTHER BRAIN REGIONS

The initial study of DARPP-32 immunoreactivity in the brain of adult rats showed its abundance in brain regions that receive a strong dopamine input (striatum, olfactory tubercle, bed nucleus

of the *stria terminalis*, *massa intercalata*, and amygdaloid nuclei; Ouimet et al., 1984). Weakly labeled neuronal cell bodies and dendrites were found in the Purkinje neurons of the cerebellum, as well as in certain glial cells, especially in the median eminence, in the arcuate nucleus, and medial habenula (Ouimet et al., 1984). In the rat cerebral cortex DARPP-32 is expressed throughout the neocortex, primarily in layer VI corticothalamic neurons (Ouimet et al., 1984; Ouimet, 1991). DARPP-32 is also found in the monkey cortex with a widespread distribution in the fetus, which becomes more restricted in the adult (Berger et al., 1990). DA signaling in the prefrontal cortex (PFC), which receives dense DA projections from the VTA, has been established to play a critical role in animals' performance in both working memory and selective attention tasks (Goldman-Rakic et al., 2000). D1R and D2R are expressed in the rat PFC, in layers VI and V respectively (Gaspar et al., 1995). The positive effects of a D1R agonist on a recognition and temporal order memory task were associated with increased phosphorylation of DARPP-32 on Thr-34 in the PFC but not in the hippocampus, whereas a D1R antagonist decreased the performance and Thr-34 phosphorylation (Hotte et al., 2006). Moreover, in mice the levels of expression in the PFC of several DA signaling related genes, including DARPP-32, were positively correlated with animals' general cognitive performance (Kolata et al., 2010).

DARPP-32 also appears to play a role in progesterone-dependent sexual behavior. Vaginal–cervical stimulation was reported to increase the number of cells exhibiting pThr-34-DARPP-32 immunoreactivity in the medial preoptic nucleus, the caudal ventromedial hypothalamic nucleus, the posterodorsal medial amygdala, and the bed nucleus of the *stria terminalis*, although the total amount of protein was not verified (Meredith et al., 1998). Progesterone enhanced DARPP-32 Thr-34 phosphorylation in the hypothalamus of female rats, independently from D1R (Mani et al., 2000). The role of DARPP-32 in the behavioral effects of progesterone was indicated by the decreased lordosis after injection of DARPP-32 antisense nucleotides in the third ventricle or in DARPP-32 knockout mice (Mani et al., 2000). In addition to its role in brain, DARPP-32 may be involved in sexual hormone action at the peripheral level since it is expressed in ovarian endocrine cells (Mayerhofer et al., 1999). Although reproductive performance is not altered in adult DARPP-32 knockout mice, progressive alterations and derangements of growth and development of ovarian follicles are observed, suggesting premature ovarian aging (Mayerhofer et al., 2004). A recent study has shown that DARPP-32 as well as other DA-related gene products, were upregulated in lateral hypothalamic orexinergic neurons by sodium deficiency (Liedtke et al., 2011). These observations suggest an evolutionary connection between the hypothalamic mechanisms of salt appetite and hedonic liking of salt taste, and possibly also mating behavior, with other D1R-regulated reward systems.

DARPP-32 IN HUMAN PHYSIOLOGY AND PATHOLOGY

DARPP-32 IN HUMAN NEUROPSYCHIATRIC DISEASES AND PSYCHOLOGICAL TRAITS

DARPP-32 mRNA is expressed in the human brain with a distribution similar to that found in monkey and rodents, with high levels in the caudate, putamen, NAc, choroid plexus, and low levels in the cerebral cortex, CA1 and CA3 regions of the hippocampus, amygdala, and Purkinje cells (Brené et al., 1994). In the PFC DARPP-32 mRNA levels increase with age (Colantuoni et al., 2008). Studies of DARPP-32 alterations in humans include measurements in post-mortem tissues using biochemical and immunohistochemical methods, as well as genetic analyses. Expression of DARPP-32 is not changed by the massive alteration in DA innervation in patients with Parkinson's disease or progressive supranuclear palsy (Girault et al., 1989b; Raisman-Vozari et al., 1990). One of the difficulties of studies of human brain samples is the variability of the levels of DARPP-32, as those of several other proteins, with the post-mortem delay, requiring careful matching of delays and large sample size (Girault et al., 1989b). Studies in rodents confirmed the independence of DARPP-32 expression levels from striatal DA innervation during development and following lesions of nigrostriatal neurons (Ehrlich et al., 1990; Raisman-Vozari et al., 1990; Brown et al., 2005). In contrast, a decreased DARPP-32 immunoblot signal was reported in the dorsolateral PFC of schizophrenic patients in comparison to matched controls (Albert et al., 2002). A decreased number of DARPP-32-positive neurons was also observed by immunohistochemistry in the superior temporal gyrus of schizophrenic patients compared to age and sex-matched control subjects (Kunii et al., 2011). Another study reported a decreased level in the dorsal PFC of schizophrenic and bipolar patients (Ishikawa et al., 2007). In contrast to these convergent results on protein levels, results concerning DARPP-32 mRNA levels were variable depending on the studies: unchanged in the dorsal prefrontal and anterior cingulate cortex of elderly schizophrenic patients (Baracskay et al., 2006), or slightly increased in the PFC of schizophrenic and bipolar patients (Zhan et al., 2010), or slightly decreased in schizophrenic patients who died by suicide (Feldcamp et al., 2008). A decreased number of leukocytes expressing DARPP-32 was also reported in schizophrenic and bipolar patients (Torres et al., 2009). Post-mortem studies in humans have intrinsic limitations and the significance of these intriguing findings is unclear. The existence of DARPP-32 alterations in both schizophrenia and bipolar disorder may not be surprising since the limits between these two entities appear less strict than initially thought, as recent studies identify common susceptibility genes (Bondy, 2011). However, both conditions are unlikely to be homogenous, and it is possible that decreased DARPP-32 levels in the cortex are meaningful only in subgroups of patients yet to be identified. Whether these changes are a cause or a consequence of the disease process also remains to be determined. It is unlikely that the changes result from chronic antipsychotic treatment since such treatment did not alter DARPP-32 levels in rodents (Grebb et al., 1990; Souza et al., 2008). In contrast, chronic treatment of rats with lithium or other antidepressants increased DARPP-32 levels in the frontal cortex (Guitart and Nestler, 1992).

PPP1R1B, the gene encoding DARPP-32, is located at 17q21, in or near a region possibly implicated in risk for schizophrenia by a meta-analysis of whole genome linkage (Lewis et al., 2003). A polymorphism in the *PPP1R1B* gene has been identified without association with schizophrenia in Chinese Han (Li et al., 2006; Hu et al., 2007) and Japanese (Yoshimi et al., 2008) populations. Another study in the USA identified a frequent *PPP1R1B*

haplotype predicting mRNA expression of DARPP-32 isoforms in post-mortem human brain (Meyer-Lindenberg et al., 2007). This haplotype was associated with enhanced performance in several cognitive tests that depend on frontostriatal function and increased frontostriatal connectivity in multimodal brain imaging. It was also associated with the risk for schizophrenia in a family based association analysis (Meyer-Lindenberg et al., 2007). Possible links between *PPP1R1B* polymorphisms and personality traits have also been studied. A single nucleotide polymorphism (C → T in intron 5) was associated to self-reports of anger in a German Caucasian population (Reuter et al., 2009). Another study conducted in healthy Chinese Han subjects found a correlation between several SNPs in *PPP1RB* and traits of personality such as harm avoidance or novelty seeking (Li et al., 2011). A particular haplotype (present in 32% of the subjects) in *PPP1RB* was linked to smoking quantity in a European-American cohort but not in an Afro-American one (Beuten et al., 2007). All these association studies are intriguing but at best correlative. Their validity will have to be confirmed by the replication of the associations in similar and different genetic backgrounds.

DARPP-32 SHORT ISOFORM AND CANCER

Both DARPP-32 and a shorter isoform lacking the 36 N-terminal residues and termed truncated DARPP-32 (t-DARPP) are over-expressed in gastric cancer (El-Rifai et al., 2002). The translation of t-DARPP-32 is initiated at an internal site, Met-37, from an mRNA encompassing an alternative first exon located within intron 1 of DARPP-32 (El-Rifai et al., 2002; **Figure 4**). This transcript has also been detected in human brain (El-Rifai et al., 2002; Meyer-Lindenberg et al., 2007). t-DARPP-32 lacks the N-terminal region involved in PP1 inhibition. The physiological role of t-DARPP-32, if any, is not known and Met-37 is not conserved in all species (e.g., it is absent in rat). In contrast t-DARPP may play an important role in human cancer cells. In gastric adenocarcinoma, t-DARPP promotes cell survival through an increase in Bcl2 levels mediated by a pathway involving activation of Akt, CREB, and ATF1 (Belkhiri et al., 2008). t-DARPP is over-expressed in breast cancer cells resistant to herceptin™, a therapeutic monoclonal antibody that blocks erbB2 (Gu et al., 2009; Hamel et al., 2009). This effect involves phosphorylation of Thr-75 and activation of Akt, and is antagonized by full length DARPP-32 (Gu et al., 2009; Hamel et al., 2009). Interestingly, the effects of t-DARPP-32 are mediated in part by activation of β-catenin-dependent transcription (Vangamudi et al., 2011). In both breast and upper gastrointestinal tract cancer cells, t-DARPP-32 appears to activate Akt upstream from phosphatidylinositol-3-kinase (Vangamudi et al., 2010, 2011). Another link between DARPP-32 and transformed phenotype has been identified in breast cancer. DARPP-32 binds to the juxtamembrane region of unstimulated discoidin domain receptor-1 (DDR1), a receptor tyrosine kinase activated by collagen (Hansen et al., 2006). Co-expression of the two proteins inhibited cell migration and filopodia formation through phosphorylation of DARPP-32 Thr-34 in response to Wnt-5a activation of cAMP production and PKA (Hansen et al., 2006, 2009). These results are interesting since they identify t-DARPP as a potential therapeutic target in cancer. They also suggest the existence of novel signaling function of this protein that remain to be elucidated at the molecular level but which might also be relevant in neurons.

CONCLUSION

DARPP-32 is one of several PKA-regulated inhibitors of PP1 and has additional functions which endow it with a large number of possible regulatory roles. DARPP-32 is highly expressed in specific cells especially in neurons, among which the striatal MSNs have the highest DARPP-32 concentration. Its phosphorylation has been thoroughly investigated and DARPP-32 is the hub of a rich network of regulations. It controls an amazingly wide variety of properties of MSNs from ion channel permeability and synaptic plasticity, to nuclear chromatin response. The combination of experimental and modeling studies suggests that in MSNs DARPP-32 is a robust integrator of signaling whose main role may be to increase the reliability in decoding the information mediated by glutamate and DA, as well as other inputs. As such it may not be essential for the basal functioning of the basal ganglia and cortex, but it may perhaps be expected to increase evolutionary fitness by improving performance in challenging conditions.

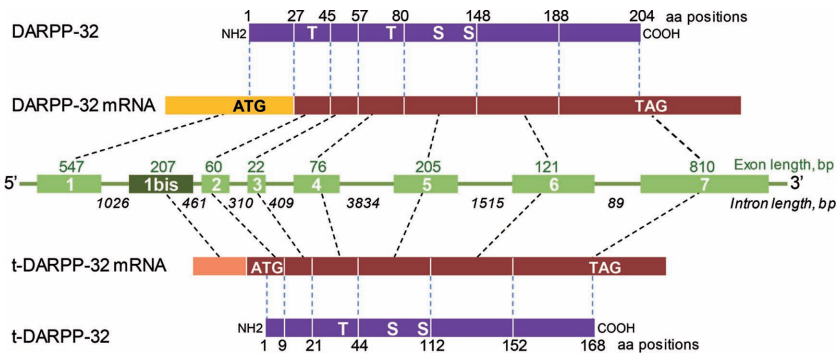

FIGURE 4 | Exon organization of human DARPP-32 and its truncated isoform. Schematic representation of the *DARPP-32* (*PPP1R1B*) gene (green, middle) with introns (green lines with length below) and exons (green boxes with number in white and length above). DARPP-32 protein and the short isoform, t-DARPP-32 are indicated in magenta. The length of exons and introns is indicated in base pairs (bp) and the borders of coding exons in amino acid residues in protein sequences. The position of major phosphorylation sites is indicated on the proteins (S, serine, T, threonine). The scheme is based on El-Rifai et al. (2002) and NCBI sequences.

Accordingly the use of a variety of sophisticated mouse models has shown that DARPP-32 mutations do not appear to have severe consequences on spontaneous behavior in laboratory conditions. In contrast these mutations alter the effects of a large number of therapeutic and abused drugs, which represent pharmacological challenges. Similarly, so far no mutation of DARPP-32 has been clearly associated with a human pathology. However, convergent evidence in mice and human suggests that DARPP-32 may be associated with general cognitive performance (Meyer-Lindenberg et al., 2007; Kolata et al., 2010). Thus, the intricate regulations provided by a regulatory molecule such as DARPP-32 may have been selected during vertebrate evolution to optimize brain functions without being absolutely necessary for any of them. It is interesting that the PPP1R1/DARPP-32 family of PP1 inhibitors seems to have originated in early Gnathostomes. This latter type of vertebrates arguably display a more complex behavioral repertoire than jawless animals (Agnathans) and PP1 inhibitors may have contributed to this evolution. In humans, DARPP-32 polymorphisms have been linked to personality traits and could predispose to psychopathological states. Thus, DARPP-32 is, in principle, an interesting target for pharmacological interventions, with possibilities for enhancing or preventing modulatory functions without altering vital mechanisms. Yet, the molecular properties of DARPP-32, mostly an unstructured protein, do not provide simple binding sites for small molecules making the design of efficient drugs a challenging task. Alteration of its level of expression through gene targeting may be a better way to exert therapeutic effects not only in neuropsychiatry, but also in cancer.

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Distinct changes in CREB phosphorylation in frontal cortex and striatum during contingent and non-contingent performance of a visual attention task

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The cyclic-adenosine monophosphate response element-binding protein (CREB) family of transcription factors has been implicated in numerous forms of behavioral plasticity. We investigated CREB phosphorylation along some nodes of corticostriatal circuitry such as frontal cortex (FC) and dorsal (caudate–putamen, CPu) and ventral (nucleus accumbens, NAC) striatum in response to the contingent or non-contingent performance of the five-choice serial reaction time task (5-CSRTT) used to assess visuospatial attention. Three experimental manipulations were used; an attentional performance group (contingent, "master"), a group trained previously on the task but for whom the instrumental contingency coupling responding with stimulus detection and reward was abolished (non-contingent, "yoked") and a control group matched for food deprivation and exposure to the test apparatus (untrained). Rats trained on the 5-CSRTT (both master and yoked) had higher levels of CREB protein in the FC, CPu, and NAC compared to untrained controls. Despite the divergent behavior of "master" and "yoked" rats CREB activity in the FC was not substantially different. In rats performing the 5-CSRTT ("master"), CREB activity was completely abolished in the CPu whereas in the NAC it remained unchanged. In contrast, CREB phosphorylation in CPu and NAC increased only when the contingency changed from goal-dependent to goal-independent reinforcement ("yoked"). The present results indicate that up-regulation of CREB protein expression across cortical and striatal regions possibly reflects the extensive instrumental learning and performance whereas increased CREB activity in striatal regions may signal the unexpected change in the relationship between instrumental action and reinforcement.

Keywords: CREB, frontal cortex, caudate–putamen, nucleus accumbens, attention, goal-directed action, arousal, instrumental contingency

INTRODUCTION

Selective and sustained attention in experimental animals (mostly rats and mice but recently also monkeys) can be measured by the five-choice serial reaction time task (5-CSRTT; Robbins, 2002) which in its basic form is akin to the continuous performance tests of Rosvold and Mirsky (Rosvold et al., 1956; Mirsky and Rosvold, 1960) much used in clinical settings to quantify attention deficit in attention deficit hyperactivity disorder (ADHD) and schizophrenia and which assesses sustained attention to a number of distinct locations over a 30-min or so test session. The task has also analogies to the 5-CSRTT (Wilkinson, 1963), in which human subjects had to continuously monitor the location of visual target stimuli over repeated trials in one of the five spatially diverse locations. The task thus has elements of continuous performance test, but also has an obvious component of selective spatial attention.

The basic 5-CSRTT essentially tests the ability of the rat to sustain spatial attention divided among five locations over a large number of trials. The attentional performance is measured by the accuracy of visual discrimination, omissions, speed of responding, and by different indices of response inhibitory control (sometimes called executive functioning) such as premature and perseverative responses (see Robbins, 2002 for a detailed description and discussion of these performance measures). Following extensive training on the 5-CSRTT, performance generally reaches high and stable levels over time, with low within and between-subject variance. These characteristics of the task are well suited for studies of neural bases of selective attention in animals and have been exploited in numerous lesion and neuropharmacological studies (for a review of these studies see Robbins, 2002; Dalley et al., 2004, 2011; Chudasama and Robbins, 2006). Few studies have attempted to correlate rats' performance during the 5-CSRTT with neurotransmission such as that of acetylcholine (ACh), noradrenaline (NE), and serotonin (5-HT; Passetti et al., 2000; Dalley et al., 2001, 2002) or metabolic activity in cortical regions (Barbelivien et al., 2001). However, despite the wealth of data implicating specific neurotransmitters in the control of different aspects of performance in the 5-CSRTT, the postsynaptic signaling mechanisms that lie behind such performance are largely unexplored.

The cyclic-adenosine monophosphate response element-binding protein (CREB) family of transcription factors are

activated via phosphorylation on its serine^{133} (S^{133}) and have been implicated in a variety of functions. CREB phosphorylation is increased by exposure to a novel environment (Vianna et al., 2000), contextual fear conditioning (Impey et al., 1998; Stanciu et al., 2001), inhibitory avoidance (Bernabeu et al., 1997), and radial and Morris maze training and performance (Mizuno et al., 2002; Porte et al., 2008). Changes in CREB phosphorylation in hippocampus or dorsal striatum have been shown to depend on whether rats use place or response strategy to solve the cross maze (Colombo et al., 2003). Sustained down-regulation in CREB function by viral vector delivery of dominant negative form of CREB (mCREB) impaired accuracy in the 5-CSRTT (Paine et al., 2009) while its over-expression in the orbitofrontal cortex increased impulsivity in a forced-choice task (Sun et al., 2010).

To study the relationship between CREB function and attention performance in the 5-CSRTT, immediately after the end the task we measured by western blot the phosphorylation of CREB protein on S^{133} (p-CREB) in prefrontal cortex (PFC), caudate–putamen (CPu), and nucleus accumbens (NAC). These brain areas were chosen as previous studies have shown that they are critically involved in aspects of rats' performance in the 5-CSRTT (Muir et al., 1996; Christakou et al., 2001, 2004; Rogers et al., 2001; Passetti et al., 2002; Chudasama et al., 2003a,b). To control for the impact of reward processes to the action–outcome associations (Dickinson and Balleine, 1994) that underlie the rats' performance in the 5-CSRTT in one group of rats we degraded the instrumental contingency inherent in the task by making the reinforcement contingent on the performance of a second animal. Therefore our experimental design had three populations of rats (see **Table 1** for a summary of behavioral procedures). The first group of rats "untrained" which served as control for extended food deprivation and the effects of training and/or performing the task, was food deprived and habituated to the same testing chamber but no stimuli were ever presented or food reward delivered. The second, an attentional performance group of rats "master" (or contingent subjects) performed the task as usual whereas the third group of rats "yoked" (or non-contingent subjects) experienced the stimuli and rewards as being earned by their corresponding master rat; while their own actions had no consequences. The action–outcome contingency was therefore maintained for the master, but severely degraded for the yoked (examples are illustrated in

Table 1 | Summary of behavioral procedures.

	Trained		Untrained
Phase 1 (40–50 sessions)	Food deprivation and training on the 5-CSRTT (100 trial or 30 min)		Food deprivation
Phase 2, 7 days, stable performance	5-CSRTT (100 trials or 30 min)	5-CSRTT (100 trials or 30 min)	Habituation (house light on; 30 min)
Phase 3, one session, experiment	5-CSRTT Master (200 trials; 30 min), $n = 7$	5-CSRTT Yoked (200 trials; 30 min), $n = 7$	Exposure (house light on; 30 min), $n = 7$

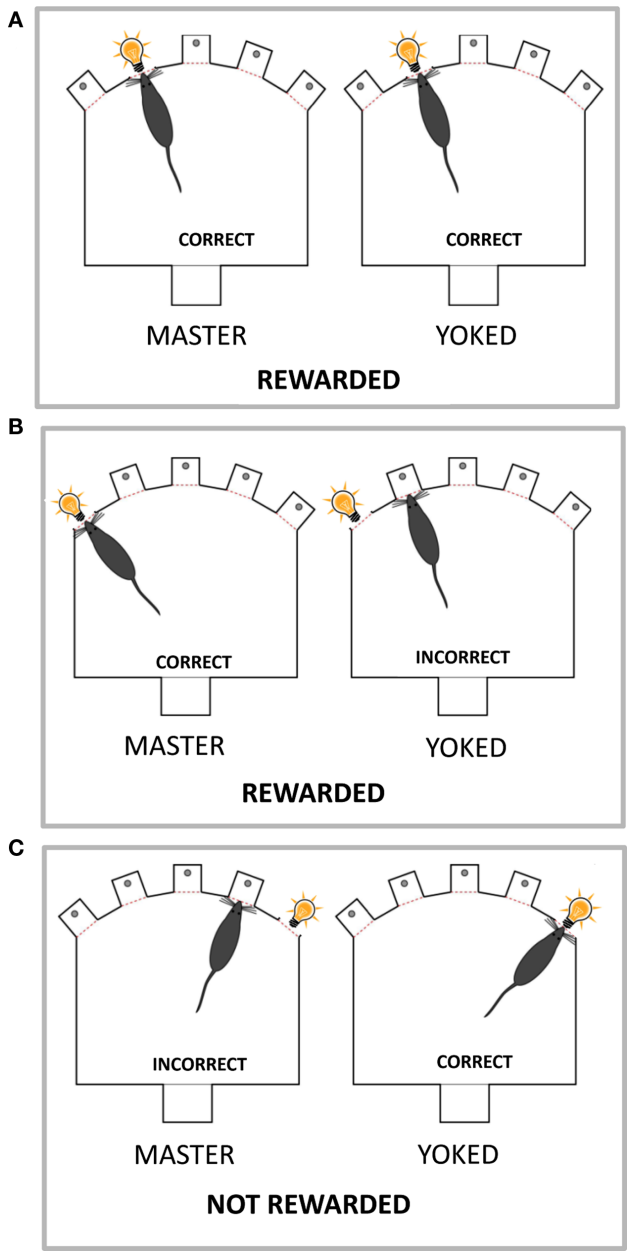

FIGURE 1 | The yoked animal receives stimuli and rewards not according to its behavior but according to the behavior of its master partner. Schematic representation of the box used to run the 5-CSRT task, with five holes set in a curved wall and a visual stimulus (light bulb; presented at random in one of the five holes for 0.5 s). One the opposite wall is set the food magazine where the earned food pellets are delivered. The master and yoked rats have three possible outcomes. **(A)** Both master and yoked rats respond in the hole with stimulus on (correct response), and both are rewarded by food. **(B)** Master rat responds in the hole with stimulus (correct response) while yoked rat responds in a non-stimulus hole (incorrect response). As master rat gained a food pellet for its correct response, the yoked rat is also rewarded by food. **(C)** Master rat makes nose poke in a non-stimulus hole (incorrect response) whereas yoked rat makes a nose poke in the stimulus hole (correct response). As master rat made an incorrect response which is not rewarded the yoked rat is not rewarded either.

Figure 1). Subjects in the yoked group could engage in the task and receive the same number of rewards as their master partners but were no longer rewarded for correct responses.

MATERIALS AND METHODS

SUBJECTS

Twenty-four male Hooded Lister rats (Charles River, Italy) were used. The rats weighed 280–320 g at the start of the experiments, and were housed in pairs until surgery, under temperature-controlled conditions (21˚C) with a day/night cycle (light on 7:00 am–7:00 pm). Limited access to food (about 15 g of Altromin pellets for rats) at the end of each day's testing kept the animals at about 90% of their free-feeding weight. Water was available *ad libitum*.

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with the national (D.L. n. 116, G.U., suppl., 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJ L 358,1, December 12, 1987; Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

BEHAVIORAL PROCEDURES

All rats were food deprived for the same period of time (about 3 months). Two groups of rats received different forms of training (see **Table 1** for a summary of behavioral procedures). The rats in the first group ($n = 7$) were habituated to the testing chamber for 7 days preceding the experimental day, but no stimuli were ever presented or food delivered; untrained controls. During these habituation sessions rats were kept with the light on in the test boxes for 30 min.

The second group of rats ($n = 14$) was trained in the 5-CSRTT. The apparatus consisted of four specially designed boxes (Campden Ins., UK) controlled on-line by Whisker software (Cambridge University Technical Services, Ltd., UK). The apparatus and all the details of training procedures have been described previously (Carli et al., 1983).

Briefly, rats were trained to wait for a fixed time (5 s) before a brief visual stimulus (0.5 s) was presented in one of the five holes. While the light was on, and for a short period afterward (limited hold), response in the hole that was illuminated (correct response) resulted in the delivery of a food pellet (45 mg Sandown Scientific, UK). Responses in the holes that had not been illuminated (incorrect responses) or failure to respond within the limited hold (omissions) caused a time-out (the house light was turned off for 2 s). Anticipatory responses (responses made before presentation of the visual stimulus during the 5-s of the waiting period) and perseverative responses (responses repeated in the holes after a correct or incorrect response but before collecting the food pellet) caused a time-out. After an anticipatory response the current trial was restarted. Each daily session consisted of 100 trials or 30 min of testing, whichever was completed sooner. Each rat had only one session per day on the 5-CSRTT.

When rats reached a stable performance with a mean of >80% correct responses, <20% omissions, at stimulus duration of 0.5 s they were assigned to two performance groups master and yoked. During the next seven training days baseline performance was recorded to ensure that both groups were evenly matched. However, only on the day of the experiment were the yoked rats actually yoked to the execution of the 5-CSRT task by their master partners. The dependent variables selected for analysis were: (a) accuracy of visual discrimination expressed as the percentage of correct responses (total correct responses/total correct + total incorrect responses × 100); (b) percentage of omissions (total omissions/total correct + total incorrect + total omissions × 100); (c) the number of premature responses in the holes during the ITI); (d) the number of perseverative responses in the holes after a correct or an incorrect response; and (e) the number of perseverative panel pushes. We also recorded (to the nearest 0.001 s) and analyzed (e) mean correct response latency; (f) mean incorrect response latency; and (g) the mean latency to collect the earned food pellet.

On the day of experiment the yoked rats were to receive the same visual stimuli and reward as their paired master subjects, but their action would lead to no programmed consequences (see **Figure 1** for a schematic representation of the contingency coupling for master and yoked subjects). As the well-trained rats complete 100 trials in less than 30 min, the number of trials during the experiment was increased to 200 to equate the time of decapitation (30 min from the start of the task) for all rats. The experiment was run on two consecutive days between 9 and 12 am. On each day four triplets of one untrained, one master, and one yoked rat were run.

SAMPLES PREPARATION

Immediately after the end of the behavioral test rats were killed by decapitation their brains quickly removed and immediately immersed in ice-cold saline for few seconds. The frontal cortex (FC; comprising prefrontal, orbitofrontal, cingulate, and primary motor cortex), CPu, and the NAC were rapidly dissected out, immediately frozen on dry ice and stored until use. Tissues were homogenized by sonication (Sonopuls, Bandelin electronic, Germany; power 30%, cycle 30%, 10–15 pulses) in 1 ml (FC and CPu) or 0.5 ml (NAC) of 1% sodium dodecyl sulfate (SDS), heated for 10 min at 95˚C and centrifuged at 5000*g* for 2 min. Supernatant fractions obtained from all tissues of interest were prepared on the same day and stored at −80˚C until they were analyzed by immunoassay. Each brain region was processed separately. Immunoblots of p-CREB, CREB, and β-actin were obtained from the same sample but run on different gels.

WESTERN BLOT ANALYSIS

The concentration of total proteins in the supernatant of each sample was measured by the bicinchoninic acid protein assay method (BCA protein assay kit, Pierce Biotechnology, USA). Equal amounts of protein extracts (20 μg) were loaded onto multi-well combs enabling 14 samples to be loaded onto the same gel. To minimize the inter-blot variability, three to four samples for each experimental group were loaded onto the same gel. In a separate well, 5 μl of dual-color pre-stained molecular mass marker (Precision Plus Protein™Standards; Bio-Rad, Italy) was loaded for molecular weight determination. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis at constant current (50 mA) and room temperature. Proteins

were transferred onto 0.45 μm pore size polyvinylidene fluoride membrane (Amersham GE Healthcare, UK) at 300 mA and 4°C for 1 h. Membranes were blocked with 5% non-fat milk in TBS-T (0.05% Tween 20 in Tris-buffered saline, pH 7.6). The blots were then incubated overnight at 4°C with primary antibodies against phospho-S^{133}CREB (anti-mouse monoclonal, 1:5000, Upstate Biotechnology, USA), CREB (anti-mouse monoclonal, 1:5000, Cell Signaling Technology, USA), and β-actin (anti-rabbit polyclonal, 1:8000, Cell Signaling Technology, USA) in TBS-T with 2% bovine serum albumin. The next day, after 5 five-min rinses with TBS-T, membranes were incubated for 1 h at room temperature with anti-mouse (1:8000 for p-CREB and CREB; Santa Cruz Biotechnology, USA) or anti-rabbit (1:10000 for β-actin; Cell Signaling Technology, USA) HRP-conjugated IgG peroxidase-labeled secondary antibodies.

The immunopositive protein bands were detected with a chemiluminescent home made ECL luminol/*p*-coumaric acid solution. Membranes were exposed in the dark room to autoradiography films (Hyperfilm ECL, Amersham GE Healthcare) then developed. Densitometric analysis of immunoblots was done to quantify the changes in protein levels using the public domain *ImageJ* (http://rsbweb.nih.gov/ij/). The optical density of the band for each antibody was linear in the range between 15 and 60 μg total protein/well as calculated by linearity tests (not showed).

STATISTICAL ANALYSIS

Differences in the number of correct responses, the number of omissions, and the number of perseverative panel pushes as well as the mean latency to collect the earned food pellet between "master" and "yoked" rats were assessed by unpaired Student's *t*-test (StatView 5.0 software). The analysis of quantitative data from western blot assays, expressed as mean percentage of untrained controls, was done by one-way ANOVA (StatView 5.0 software) and between groups comparison made by Tukey's test.

To account for changes in total CREB, p-CREB levels were expressed as the ratio of p-CREB/CREB optical densities. Then, values obtained in untrained, master, and yoked rats were divided by mean values of untrained rats. These ratios (untrained, U/U; master, M/U; and yoked, Y/U) were analyzed by one-way ANOVA followed by Tukey's test, which compared the task condition means between them.

RESULTS

BEHAVIOR OF MASTER AND YOKED RATS IN THE 5-CSRTT

When a rat perform an action for a rewarding outcome it does so via several psychological mechanisms one being "goal-directed" action analogous to the human concept of intention. Thus when the rat in the 5-CSRTT responds to a visual stimulus, among several reasons it may do it because it has learned the contingency between its action and the outcome. With extended training necessary for the optimal performance in the 5-CSRTT, actions that were originally "goal-directed" could become "automatic" and habitual. Habits (i.e., stimuli evoke directly a motor response) are relatively inflexible and require fewer cognitive resources that goal-directed actions. We predicted that if the rats were not responding habitually during the 5-CSRTT the yoked rats were expected to swiftly shift their responses from responding in the stimulus holes to the food source.

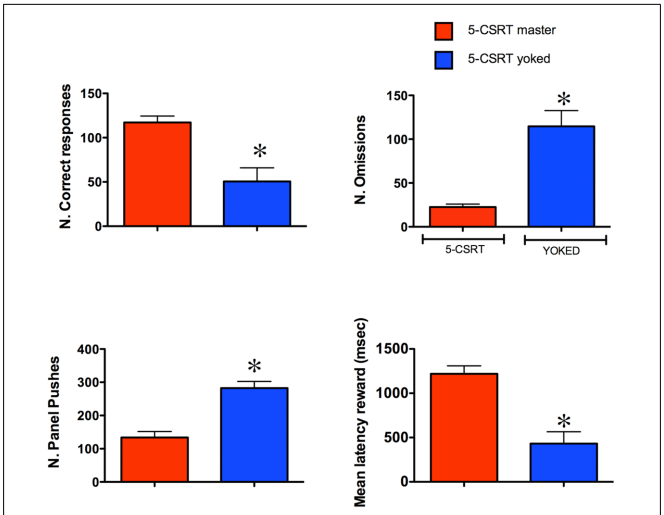

FIGURE 2 | The behavioral performance of master and yoked rats in the 5-CSRT task. The histograms represent mean ± SEM ($n = 7$ per group). The stimulus duration and the inter-trial-interval were 0.5 and 5 s respectively. * $P < 0.05$ vs. master; Unpaired Student's *t*-test.

Figure 2 shows the comparative effects of degrading the instrumental contingency on the behavior of master and yoked subjects on the 5-CSRTT. Analysis of total number of correct responses revealed that yoked rats made fewer correct responses and a higher number of omissions than master subjects (both $P < 0.05$; unpaired Student's *t*-test). Magazine entries (panel pushes) were almost twice as frequent in yoked animals compared to master subjects ($P < 0.05$; unpaired Student's *t*-test) and yoked animals collected the reward much faster than master rats ($P < 0.05$; unpaired Student's *t*-test). However, on trials in which yoked subjects made a response, they did it as accurately (83.4 ± 4.3% correct), as their master partners (86.7 ± 1.2% correct), and with similar correct response latency (master 0.72 ± 0.03 s; yoked 0.66 ± 0.03). Overall, these behavioral data show that yoked subjects very rapidly extinguished responding for target stimuli and that they directed their activity toward the food source (magazine).

p-CREB AND CREB EXPRESSION IN UNTRAINED AND MASTER AND YOKED RATS

Figure 3 shows the intensity of CREB and p-CREB signal in immunoblots obtained from the FC, CPu, and NAC of representative untrained (U) master (M) and yoked (Y) subjects.

The mean values of p-CREB and CREB protein levels, expressed as percentages of untrained control rats are shown in **Figure 4**. We found a statistically significant differences in p-CREB (FC, $F_{2,17} = 10.3$ $P < 0.001$; CPu, $F_{1,8} = 5.5$ $P < 0.001$; NAC, $F_{2,13} = 48{,}7$ $P < 0.0001$) and CREB levels (FC, $F_{2,18} = 18.3$ $P < 0.0001$; CPu, $F_{2,16} = 22.1$ $P < 0.0001$; NAC, $F_{2,18} = 73.0$ $P < 0.0001$) in master and yoked compared to untrained controls across the corticostriatal regions examined. p-CREB levels in the FC of master and yoked rats were significantly higher than in untrained rats ($P < 0.05$ vs. untrained; Tukey's test), reaching about 300% in both groups. p-CREB levels were undetectable or strongly reduced in the CPu of master rats. By contrast, p-CREB

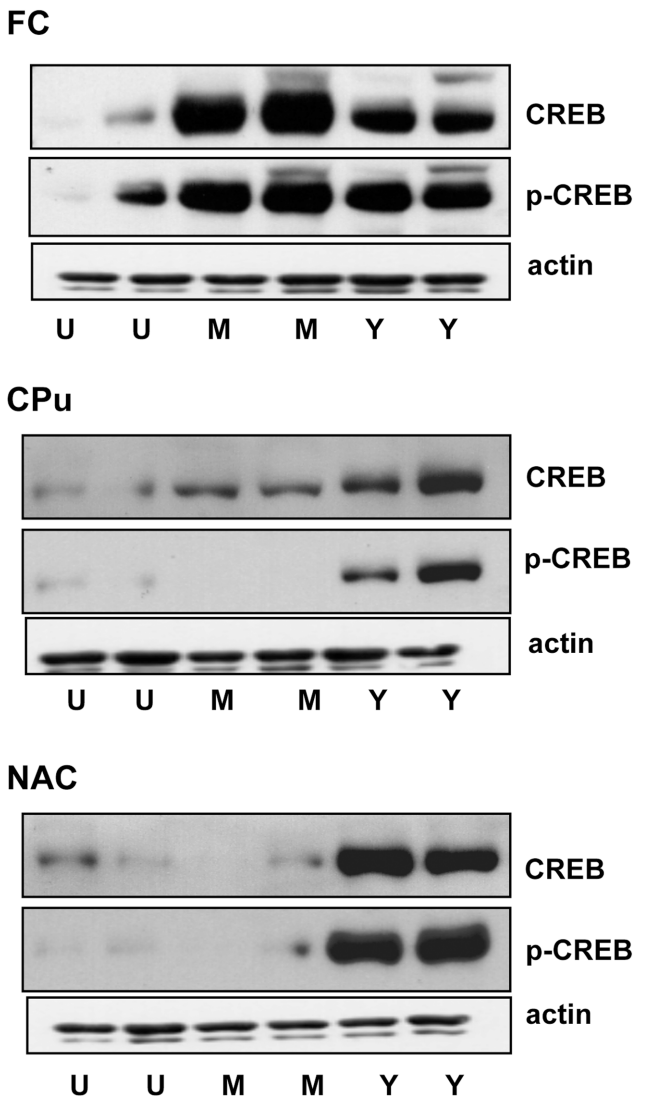

FIGURE 3 | Immunoblots of p-CREB, CREB, and actin in the frontal cortex (FC) Caudate–putamen (CPu) and nucleus accumbens (NAC) of representative untrained (U), master (M), and yoked (Y) rats. Blots were obtained from the same gel.

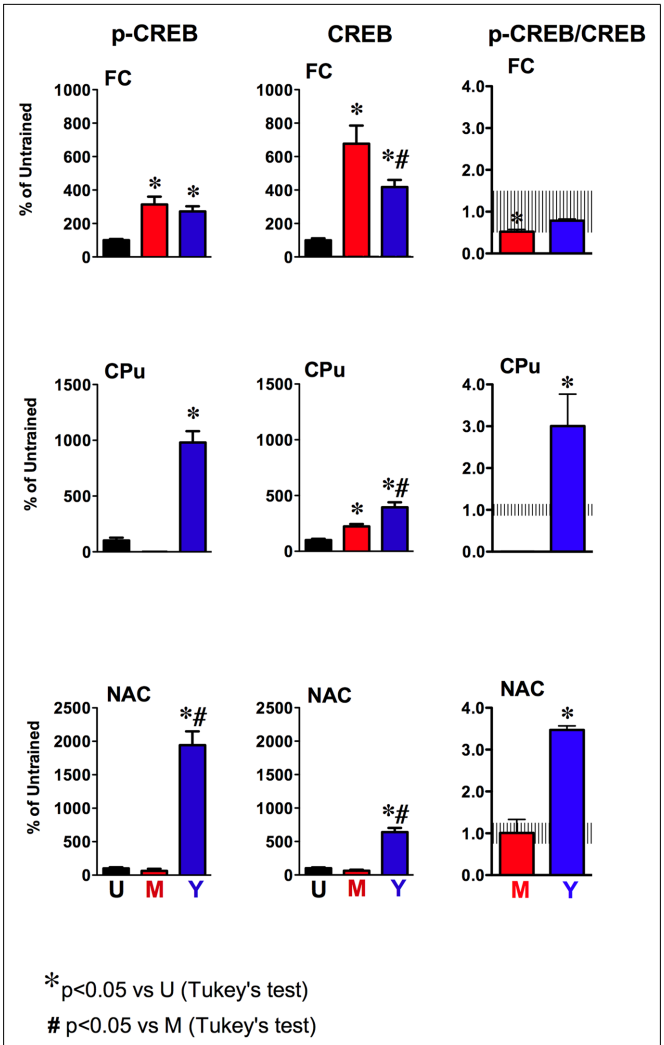

FIGURE 4 | Quantification of p-CREB and CREB levels and p-CREB/CREB ratios in the frontal cortex (FC) Caudate–putamen (CPu) and nucleus accumbens (NAC) of untrained (U), master (M), and yoked (Y) rats. Data are expressed as the percentage of protein level in untrained control rats. Histograms represent mean ± SEM of six to seven rats per group. The shaded areas represent the mean ± SD of p-CREB/CREB ratios for untrained control rats. *$P < 0.05$ vs. untrained (U); #$P < 0.05$ vs. master (M); Tukey's test.

increased by about 10-folds in the CPu of yoked rats ($P < 0.05$ vs. untrained; Tukey's test). The increase in p-CREB levels was even larger in the NAC of yoked rats, reaching almost 2000% of untrained controls ($P < 0.05$ vs. untrained and master; Tukey's test), while no significant changes were observed in the NAC of master rats.

Total CREB (unphosphorylated plus phosphorylated) levels were significantly increased in the FC and CPu of master rats (both $P < 0.05$ vs. untrained; Tukey's test) whereas no significant changes were observed in the NAC. In yoked rats, CREB increased in all brain regions examined. The effect was stronger in the NAC, reaching about 700% ($P < 0.05$ vs. untrained or master rats; Tukey's test). In the FC of yoked rats CREB levels increased by 400% and this increase was significantly less than in master rats (700%; $P < 0.05$ vs. master; Tukey's test) whereas in the CPu the increase in CREB signal was larger in yoked compared to master rats ($P < 0.05$; Tukey's test). In all brain regions examined, actin levels (**Figure 3**) did not change across experimental groups.

The ratios of p-CREB/CREB are reported in **Figure 4**. The mean ± SD ratio for untrained rats was 1.00 ± 0.54 in FC; 1.00 ± 0.14 in CPu and 1.00 ± 0.25 in NAC and are shown as the shaded areas in **Figure 4**. One-way ANOVA revealed significant differences across groups in p-CREB/CREB ratios in the PFC ($F_{2,18} = 3.5$, $P = 0.05$) CPu ($F_{1,8} = 243.4$, $P < 0.0001$) and NAC ($F_{2,13} = 83.0$, $P < 0.0001$). *Post hoc* analysis showed that p-CREB/CREB ratio in master rats was actually decreased to about 52% of untrained rats ($P < 0.05$; Tukey's test) whereas that

of yoked rats was 73% of untrained controls ($P > 0.05$; Tukey's test). The p-CREB/CREB ratio of master and yoked rats was not significantly different ($P > 0.05$; Tukey's test). The ratio p-CREB/CREB was strongly increased in the CPu (+200%) and NAC (+248%) of yoked rats (both $P < 0.05$ vs. untrained; Tukey's test). No significant changes were observed in the NAC of master rats. The ratio p-CREB/CREB in the CPu of master rats was not calculated because p-CREB levels were not detectable in most samples.

DISCUSSION

This study is the first to measure p-CREB and CREB protein levels along different nodes of prefrontal corticostriatal circuitry in rats that performed a visuospatial attentional task such as 5-CSRTT. The main finding was that rats performing the 5-CSRTT were able to detect instrumental contingency change and adapt the performance accordingly suggesting that their performance was goal-directed and not "automatized" or habitual (Balleine and Dickinson, 1998; Yin et al., 2008). The behavioral changes in subjects experiencing a non-contingent reward (yoked rats) were associated with increased CREB phosphorylation in all areas examined. In contingent subjects (master rats), who performed the 5-CSRTT as usual, to an increase in p-CREB in the FC corresponded a decrease in the CPu and no changes in the NAC. Compared to food-deprived untrained control rats, never exposed to the 5-CSRTT training, there was a substantial increase in total CREB protein levels in master and yoked rats across all areas examined save in the NAC of master rats, suggesting that sustained behavioral experience may increase CREB protein synthesis. These data indicate an anatomical dissociation of psychological functions and their underlying CREB signaling mechanisms that guide rats' performance in the 5-CSRTT.

Master rats performed the task with high efficiency missing only few stimuli. By contrast yoked rats' performance toward the target stimuli was rapidly extinguished as shown by substantial decrease in the number of correct responses and an increase in the number of responses directed toward the food source, which were also much faster than in master rats. This switch in responding from nose-pokes in stimulus holes to panel pushes is an indication of rats' ability to shift their attention to a more reliable predictor of reward. In the FC of master and yoked rats p-CREB was increased to the same level. However, these changes were largely compensated by the up-regulation of total CREB in both master and yoked compared to untrained rats indicating that there was not a real increase in p-CREB as shown by p-CREB/CREB ratios. Thus, despite the divergent behavior in master and yoked rats, their p-CREB/CREB ratios were not substantially different implying no behavioral selectivity of CREB activation in this brain region. Functional specialization of frontocortical regions and corticostriatal circuits has been described for the 5-CSRTT and thus it could not be excluded that various aspects or types of performance might affect CREB function differentially in various frontocortical regions (Muir et al., 1996; Christakou et al., 2001, 2004; Rogers et al., 2001; Passetti et al., 2002; Chudasama et al., 2003b). It should be noted that we cannot distinguish the CREB activity in these specialized PFC regions as we have measured CREB function in the frontal pole comprising PFC, orbitofrontal, cingulate, and pre-motor areas and thus our measure reflects the resultant activity in this cortical area.

The most compelling finding of this investigation is that of differences in CREB activation across the FC, CPu, and NAC between yoked and master rats. This finding specifies the conditions that engage this signal transduction system in these brain areas. Thus increased p-CREB/CREB ratios in the CPu and NAC of yoked subjects may signal a mismatch between action and reinforcement. The resulting shift in attention from responding to stimuli that are no more predictive of reward to those perceived as more reliable predictors of the reinforcement (i.e., panel pushes) may be an important mechanism that promotes the new contingency learning in yoked rats. CREB has been shown to positively regulate dorsal striatum-dependent synaptic plasticity, procedural learning, and specific cognitive response-based behavioral strategies (Colombo et al., 2003; Pittenger et al., 2006).

It is interesting to note that rats bearing selective lesions of the NAC are still able to detect the change in the action–outcome contingency (Balleine and Killcross, 1994; Corbit et al., 2001) thus suggesting that NAC is not necessary for instrumental contingency encoding. Based on traditional two-process theory (Rescorla and Solomon, 1967) that stimuli, which predict an outcome can modulate animals' affective arousal, Balleine and Killcross (1994) have argued that NAC is not directly involved in integrating the information that mediate instrumental action but plays a central role in the way conditioned affective arousal is expressed in performance. Increased CREB activity in the NAC enhances NMDA receptor mediated synaptic transmission (Dong et al., 2006; Huang et al., 2008) and decreases animals' responses to emotionally important stimuli (Barrot et al., 2002). In analogy to finding that activation of CREB in the NAC helps to limit the enhanced responsiveness to repeated cocaine exposure (Carlezon et al., 1998; Pliakas et al., 2001), it could be hypothesized that the increase in p-CREB in the NAC may operate to dampen the negative consequences on behavior by increased levels of arousal induced by the shift in contingency. Interestingly, Christakou et al. (2004) found that the mPFC/NAC systems, which do not appear to be involved in the control of executive attention, integrate the information about the consequences of action in relation to anticipated reward and thus modulate the affective/arousing aspects of the 5-CSRTT performance.

The difference in p-CREB/CREB ratios in the CPu and NAC in master and yoked rats is unlikely to be due to some difference in the level of satiation or consummatory behavior as yoked rats received the same number of food pellets as their master partners. It could be argued that the contingent performance of master rats required a greater degree of executive control to coordinate the behavioral sequence necessary to obtain the reinforcement, which included visual search, detection, and response selection as well as tight organization of motor activity "runs" between the magazine and response holes located on the opposite walls of the operant chamber. Although it cannot be excluded that changes in p-CREB in yoked rats may reflect at least in part the reduced number of stimulus bound response sequences necessary to obtain the reward increased not decreased p-CREB was positively associated with motor activity (Giordano et al., 2010).

The opposite changes in CREB activity in cortical and striatal regions may reflect differences in molecular signals of frontocortical vs. striatal neurons. Reciprocal regulation of cortical vs. striatal regions is well established, for example for neurotransmitters such as dopamine (Wilkinson et al., 1997); therefore it is unsurprising to find evidence of reciprocal changes in molecular signals in these brain regions. Significant differences in p-CREB between FC and CPu in response to electroconvulsive seizures have been reported (Tanis et al., 2008). No changes in p-CREB/CREB ratio were detected in the NAC of master rats whereas in the CPu of master rats p-CREB/CREB was not calculated because p-CREB signal was abolished in most samples. This dissociation of CREB activation in the CPu and NAC of contingent subjects is interesting as mPFC/CPu but not mPFC/NAC appears to control executive attention in the 5-CSRTT (Christakou et al., 2001, 2004).

Compared to untrained controls master and yoked rats which had similar training in the 5-CSRTT procedure had higher levels of total CREB in FC and CPu as well as in the NAC. The lack of changes in total ERK in the same brain areas suggests that there may be some specificity in CREB changes (M. Carli, Unpublished results). The increase in CREB levels possibly reflects instrumental action–outcome learning in this task's procedure. It is worth noting that optimal performance in the 5-CSRTT requires a complex and tightly timed response sequence, which is achieved only after extensive training (2–3 months). As p-CREB and total CREB levels were measured immediately after the behavioral session we are unable to distinguish the effects of instrumental learning from actual task performance. However, it is possible that up-regulation of CREB protein may pre-exist as a result of instrumental learning. Indeed, some studies have shown that there is a spatio-temporal dynamic in CREB activity during learning (Porte et al., 2008) and that consolidation of instrumental performance requires protein synthesis (Hernandez et al., 2002). In analogy to what observed with visual experience (Cancedda et al., 2003) it could be hypothesized that the extensive behavioral experience (i.e., acquisition of instrumental performance) may have increased CRE-dependent genes expression and subsequent CREB synthesis (Meyer and Habener, 1993).

As untrained controls were food deprived and exposed to the operant boxes (but never trained on the 5-CSRTT) it is unlikely that this increase in CREB is due to food deprivation or to exposure to the contextual cues in the operant boxes.

The lack of an increase in total CREB levels in the NAC of master rats is somewhat surprising as CRE-mediated transcription within NAC is regulated by environmental stimuli and protein synthesis in the NAC is necessary for instrumental learning (Hernandez et al., 2002; Huang et al., 2008). This finding would suggest some effect of non-contingent performance on CREB protein levels. In the NAC of yoked rats there was a 20-fold increase in p-CREB, which in turn may induce its own gene transcription (Meyer and Habener, 1993) although inhibition of CREB protein turnover may have also contributed. However, CREB is a 43-kDa protein and a *de novo* synthesis in about 30 min of a such a big protein is unlikely. The antibody used to detect CREB protein levels does not distinguish between unphosphorylated and phosphorylated CREB thus it is likely that the total CREB levels may reflect at least in part the increase in p-CREB.

These data lend additional information to the view that cortical and striatal functions determine the sensitivity of behavior to its consequences (Balleine and Dickinson, 1998; Corbit and Balleine, 2003; Killcross and Coutureau, 2003; Yin et al., 2005, 2008; Lex and Hauber, 2009, 2011). Consistent with this view are also human imaging data showing that action–reward contingency computations activate neural responses in a network of brain regions including the ventromedial PFC, dorsomedial striatum, and the inferior frontal gyrus (Tanaka et al., 2008; Balleine and O'Doherty, 2010; Liljeholm et al., 2011). It is also of particular interest that in rats performing the 5-CSRTT cortical noradrenaline release showed sustained elevations only when contingency was degraded (Dalley et al., 2001). Although the effects of increased neural activity on CREB phosphorylation in corticostriatal circuits has yet to be determined burst of neural activity has been shown to increase p-CREB in hippocampal neurons (Bito et al., 1996). On the other hand, over-expression of CREB in slice culture of the NAC and locus coeruleus (the main source of NE projection to the cortex) neuronal cell types increased membrane excitability (Dong et al., 2006; Han et al., 2006; Huang et al., 2008) whereas decreased CREB activity in the NAC correlated with increased expression of certain K^+ channels and reduced electrical excitability of NAC neurons (Wallace et al., 2009). Thus it could not be excluded that these differential changes in p-CREB along the nodes of corticostriatal circuits may reflect differences in neuronal activity driven by contingent or non-contingent performance.

In summary the data show that increased CREB activity in striatal regions of yoked rats may signal the unexpected change in the relationship between the instrumental action and reinforcement. In addition, it is possible that up-regulation of CREB protein across cortical and striatal subregions may reflect the extensive instrumental performance acquisition.

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L-DOPA-induced dyskinesia and abnormal signaling in striatal medium spiny neurons: focus on dopamine D1 receptor-mediated transmission

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Dyskinesia is a serious motor complication caused by prolonged administration of L-DOPA to patients affected by Parkinson's disease. Accumulating evidence indicates that L-DOPA-induced dyskinesia (LID) is primarily caused by the development of sensitized dopamine D1 receptor (D1R) transmission in the medium spiny neurons (MSNs) of the striatum. This phenomenon, combined with chronic administration of L-DOPA, leads to persistent and intermittent hyper-activation of the cAMP signaling cascade. Activation of cAMP signaling results in increased activity of the cAMP-dependent protein kinase (PKA) and of the dopamine- and cAMP-dependent phosphoprotein of 32 kDa (DARPP-32), which regulate several downstream effector targets implicated in the control of the excitability of striatal MSNs. Dyskinesia is also accompanied by augmented activity of the extracellular signal-regulated kinases (ERK) and the mammalian target of rapamycin complex 1 (mTORC1), which are involved in the control of transcriptional and translational efficiency. Pharmacological or genetic interventions aimed at reducing abnormal signal transduction at the level of these various intracellular cascades have been shown to attenuate LID in different animal models. For instance, LID is reduced in mice deficient for DARPP-32, or following inhibition of PKA. Blockade of ERK obtained genetically or using specific inhibitors is also able to attenuate dyskinetic behavior in rodents and non-human primates. Finally, administration of rapamycin, a drug which blocks mTORC1, results in a strong reduction of LID. This review focuses on the abnormalities in signaling affecting the D1R-expressing MSNs and on their potential relevance for the design of novel anti-dyskinetic therapies.

Keywords: cAMP, dopamine- and cAMP-regulated phosphoprotein 32 kDa, extracellular signal-regulated protein kinases, immediate early genes, mammalian target of rapamycin, Parkinson's disease

The striatum, which includes the caudate–putamen and the nucleus accumbens, is the major component of the basal ganglia, a set of interconnected subcortical structures critically involved in motor control. The GABAergic medium spiny neurons (MSNs) are the principal neuronal type in the striatum and represent the main receiving station of the basal ganglia. Their large dendritic arborization is innervated by excitatory glutamatergic inputs from cortical, thalamic, and limbic areas and modulatory dopaminergic inputs from midbrain neurons located in the substantia nigra pars compacta (SNc) and the ventral tegmental area.

In the caudate–putamen, which corresponds to the dorsal part of the striatum, dopamine regulates the activity of MSNs by acting on dopamine D1 receptors (D1Rs) and dopamine D2 receptors (D2Rs). It has become clear that D1Rs and D2Rs have a very distinct pattern of expression in the two projection pathways which connect the striatum to the output nuclei of the basal ganglia (i.e., substantia nigra pars reticulata and internal segment of the globus pallidus). In particular, it has been shown that D1Rs are located in the MSNs which directly innervate the substantia nigra pars reticulata and the internal segment of the globus pallidus. In contrast, D2Rs are present in the MSNs which project to these nuclei indirectly, via a circuit including the external segment of the globus pallidus and the subthalamic nucleus (Gerfen et al., 1990; Gerfen, 1992; Gong et al., 2003; Valjent et al., 2009).

The above distinction is at the basis of a commonly accepted model of basal ganglia transmission, which proposes that the activation of the neurons of the "direct" striatonigral pathway facilitates motor activity via disinhibition of thalamo-cortical neurons, whereas activation of the neurons of the "indirect" striatopallidal pathway reduces motor activity by increasing inhibition on thalamo-cortical neurons (Albin et al., 1989; Alexander and Crutcher, 1990; DeLong, 1990). The same model also posits that dopamine promotes motor activity by increasing the activity of striatonigral MSNs and, concomitantly, by inhibiting striatopallidal MSNs (Albin et al., 1989; Gerfen et al., 1990). These contrasting actions of dopamine depend on the selective expression of D1Rs and D2Rs in striatonigral and striatopallidal MSNs, as well as on the coupling of these receptors to different G-proteins. Thus, activation of D1Rs leads to Gαolf-mediated stimulation of adenylyl cyclase and increased cAMP, whereas activation of D2Rs leads to Gαi/o-mediated inhibition of adenylyl cyclase (Stoof and Kebabian, 1981; Herve et al., 1993; Zhuang et al., 2000).

Striatal MSNs are targeted by a large number of drugs, including addictive substances and antipsychotic drugs. Moreover, MSNs are

the main target of medications used for the treatment of Parkinson's disease (PD), a neurodegenerative disorder characterized by the progressive loss of the dopaminergic neurons of the SNc (Hornykiewicz, 1963; Braak et al., 2003). PD is commonly treated with the dopamine precursor L-DOPA, which efficiently counteracts the motor symptoms of the disease, i.e., rigidity, tremor, and hypokinesia (Cotzias et al., 1967; Birkmayer and Hornykiewicz, 1998). However, prolonged administration of L-DOPA results in the appearance of choreic, dystonic, and ballistic movements, collectively referred to as L-DOPA-induced dyskinesia (LID). These involuntary movements represent a serious limitation to the current pharmacotherapy for PD, particularly during the advanced stages of the disease (Obeso et al., 2000; Fabbrini et al., 2007).

The need for a therapy able to efficiently counteract LID has led to considerable progress in the understanding of the molecular basis of this motor disorder. In particular, striatal MSNs have become the subject of intense investigations, which identified a number of molecular abnormalities implicated in dyskinetic behavior. These abnormalities are primarily linked to the loss of dopamine input to the basal ganglia, which strongly enhances the responsiveness of MSNs to dopaminergic drugs. Such a sensitization is especially evident in the case of L-DOPA, which, following interruption of the nigrostriatal dopaminergic input, acquires the ability to affect multiple signaling pathways. One of the main goals of current research on LID is to study abnormal signaling processes induced by L-DOPA in the dopamine-depleted striatum and to determine their involvement in the emergence of pathological motor behavior.

Accumulating evidence indicates that LID develops in response to activation of sensitized D1Rs located on the MSNs of the direct striatonigral pathway. The effects produced by L-DOPA in these neurons are not limited to hyper-activation of the canonical cAMP/dopamine- and cAMP-regulated phosphoprotein of 32 kDa (DARPP-32) cascade, but include modifications of signaling pathways not typically related to dopaminergic transmission, such as the extracellular signal-regulated kinases (ERK) and the mammalian target of rapamycin (mTOR) cascades. The present review focuses on these signaling abnormalities and on their potential relevance for the treatment of LID.

MECHANISMS OF D1R SENSITIZATION IN PD: ALTERATIONS IN RECEPTOR DISTRIBUTION, G-PROTEIN COUPLING, AND ADENYLYL CYCLASE EXPRESSION

In simple terms, the development of sensitized D1R transmission in PD can be regarded as a compensatory response to the lack of dopamine in the striatum. However, a clear understanding of the mechanisms implicated in this phenomenon has been elusive. Studies using 6-hydroxydopamine (6-OHDA) and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), two toxins used to generate experimental models of PD, indicate that the number and affinity of D1Rs is unchanged following dopamine depletion (Breese et al., 1987; Savasta et al., 1988; Marshall et al., 1989; Joyce, 1991; Aubert et al., 2005). Similar results were obtained in postmortem samples from parkinsonian patients (Pimoule et al., 1985; Shinotoh et al., 1993; Hurley et al., 2001). However, work performed in non-human primates indicates that the loss of dopaminergic input to the striatum results in increased recruitment of D1Rs at the plasma membrane of MSNs (Guigoni et al., 2007), a phenomenon of potential relevance for the induction of LID.

Studies performed in MPTP lesioned monkeys have shown that repeated administration of L-DOPA increases the binding of the D1R antagonist SCH23390 to striatal membranes (Aubert et al., 2005). This effect is not accompanied by changes in the expression of D1R mRNA, suggesting that it may depend on alterations in the number of functionally available receptors. In support of this possibility, it has been shown that, in rodent and non-human primate models, LID is associated with increased localization of D1Rs at the cell surface, which may be caused by impaired receptor internalization and trafficking (Guigoni et al., 2007; Berthet et al., 2009).

The pathological enhancement in the number of D1Rs at the plasma membrane is likely to contribute to the increase in D1R transmission associated to LID and represents a potential target for therapeutic interventions. In line with this idea, recent findings have shown that LID is reduced by promoting G-protein coupled receptor (GPCR) desensitization. This process starts with the phosphorylation of the receptor by GPCR kinases (GRKs), followed by binding to arrestins and receptor internalization (Shenoy and Lefkowitz, 2003). Experiments performed in 6-OHDA-lesioned rats and MPTP lesioned monkeys show that lentiviral-mediated overexpression of the GRK6 in the striatum attenuates LID (Ahmed et al., 2010). In contrast, dyskinesia is worsened when GRK6 expression is reduced by transfection with a GRK6 miRNA. Moreover, GRK6 overexpression does not interfere with the anti-akinetic action of L-DOPA and may therefore represent a potential strategy to control dyskinetic behavior (Ahmed et al., 2010).

In cortical neurons, activation of the transcription factor cAMP response element-binding protein (CREB) is known to increase the levels of brain-derived neurotrophic factor (BDNF), which is involved in activity-dependent synaptic plasticity (Shieh et al., 1998; Tao et al., 1998). Interestingly, repeated administration of L-DOPA promotes BDNF expression in the frontal cortex of 6-OHDA-lesioned rats, via activation of dopamine D1-type receptors (Guillin et al., 2001). This effect, in concomitance with 6-OHDA-induced overexpression of BDNF TrκB receptors, is thought to increase the levels of dopamine D3 receptors (D3Rs) in the striatum (Guillin et al., 2001). Enhanced D3R expression in the MSNs of the direct pathway is associated to LID (Bordet et al., 1997, 2000; Guillin et al., 2001; Bezard et al., 2003) and may play a role in this condition by further exacerbating sensitized D1R transmission. In fact, D3Rs have been shown to exert a synergistic effect on D1R-mediated transmission through direct intramembrane interaction (Fiorentini et al., 2008; Marcellino et al., 2008). In support of this possibility, co-treatment with L-DOPA and the D3R antagonist ST 198 restores normal levels of membrane-bound D1Rs in dyskinetic animals (Berthet et al., 2009) and counteracts dyskinesia in experimental models of PD (Bezard et al., 2003; Kumar et al., 2009; Visanji et al., 2009; but see also Mela et al., 2010).

The increased recruitment of D1Rs at the plasma membrane may also be responsible for the increase in coupling of striatal D1Rs to Gαolf protein, which has been described in the striata of dyskinetic monkeys (Aubert et al., 2005). In connection to

this observation, studies performed in 6-OHDA-lesioned rats and in postmortem samples from parkinsonian patients have demonstrated that loss of striatal dopamine is accompanied by increased levels of Gαolf (Herve et al., 1993; Corvol et al., 2004; Rangel-Barajas et al., 2011). However, Gαolf overexpression subsides during chronic L-DOPA administration (Corvol et al., 2004; Rangel-Barajas et al., 2011) and striatal levels of Gαolf in dyskinetic mice are similar to those of control animals (Rangel-Barajas et al., 2011).

Striatal MSNs express high levels of adenylyl cyclase type 5 (Glatt and Snyder, 1993; Mons and Cooper, 1994), which is stimulated in response to D1R-mediated activation of Gαolf (Herve et al., 1993; Zhuang et al., 2000). Evidence obtained using 6-OHDA-lesioned rats shows that dopamine depletion increases the levels of adenylyl cyclase type 5 in the striatum (Rangel-Barajas et al., 2011). A similar increase is also observed in the substantia nigra pars reticulata, which is innervated by the MSNs of the direct pathway (cf. above; Rangel-Barajas et al., 2011). Interestingly, these effects are maintained during repeated administration of L-DOPA, but only in animals displaying severe dyskinesia (Rangel-Barajas et al., 2011).

In summary, the work described above indicates that LID is associated to increased recruitment of D1Rs at the cell surface and to overexpression of adenylyl cyclase type 5 in the striatal MSNs of the direct pathway (**Figure 1**). These modifications are likely to influence dopaminergic transmission in the striatum and may underlie the enhancement in the ability of L-DOPA to increase the levels of cAMP, thereby activating cAMP-dependent protein kinase (PKA). The importance of augmented PKA activity in dyskinesia is indicated by the observation that, in 6-OHDA-lesioned rats, striatal infusion of the PKA inhibitor Rp-cAMPS attenuates LID (Lebel et al., 2010). The following sections will focus on the effects produced by L-DOPA-induced activation of the cAMP/PKA cascade and on their potential relevance for the development and expression of LID.

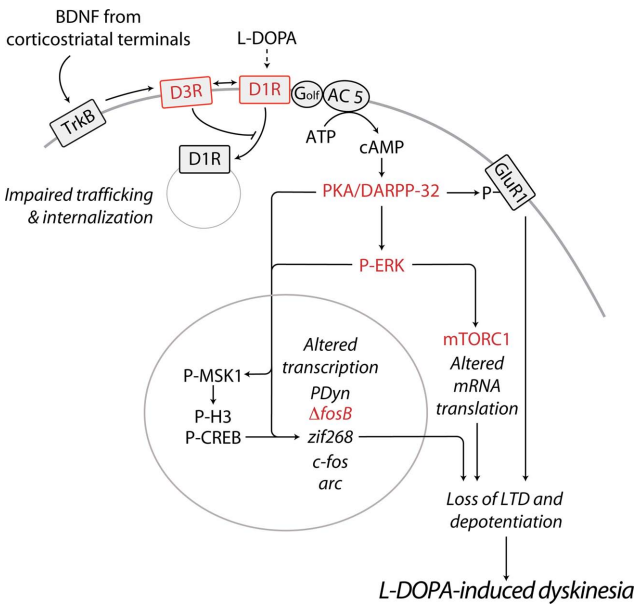

FIGURE 1 | Schematic diagram illustrating some of the major abnormalities related to sensitized D1R-signaling and associated to LID. In PD, the loss of striatal dopamine leads to sensitization of D1Rs on the striatonigral MSNs of the direct pathway. Emerging evidence indicates that, if persistent, this phenomenon results in the appearance of dyskinesia. D1R sensitization may be caused by augmented D1R expression at the cell surface. Chronic administration of L-DOPA promotes the release of BDNF from corticostriatal neurons, leading to activation of TrκB receptors and increased expression of D3Rs, specifically in striatonigral MSNs. Direct interaction with D3Rs is likely to increase the levels of membrane-bound D1Rs, thereby exacerbating D1R sensitization and dyskinetic behavior. In line with this possibility, D3R antagonists have been found to counteract LID in experimental models of PD. Sensitized D1R transmission may also be caused by increased levels of adenylyl cyclase 5 (AC 5) in striatonigral MSNs. Increased responsiveness of the D1R/Gαolf/AC5 machinery to L-DOPA results in augmented synthesis of cAMP and hyper-activation of PKA and DARPP-32. Pharmacological inhibition of PKA, or genetic inactivation of DARPP-32 have been shown to reduce LID. Abnormal PKA/DARPP-32 signaling increases the phosphorylation of GluR1. This effect promotes the excitability of MSNs and may participate in the loss of corticostriatal LTD and depotentiation associated to LID. Sensitized D1R-mediated transmission leads also to activation of ERK, which controls transcriptional and translational processes. Both pharmacological and genetic suppression of ERK signaling counteracts the development and expression of LID. In the nucleus, PKA/DARPP-32 and ERK/MSK1 signaling leads to phosphorylation of CREB and histone H3, and increased expression of immediate early genes and prodynorphin. Reduced expression/activity of Δ*fosB* efficiently counteracts LID. Activation of ERK promotes mTORC1-dependent signaling, thereby accelerating mRNA translation. Blockade of mTORC1 with rapamycin has been found to attenuate the development of LID. Red color indicates receptors or signaling components whose targeting reduces LID. See text for abbreviations.

DOWNSTREAM OF cAMP AND PKA: ROLE OF DARPP-32 IN DYSKINESIA AND ABNORMAL CORTICOSTRIATAL DEPOTENTIATION

Dopamine D1 receptor-mediated transmission in striatal MSNs depends not only on PKA-dependent phosphorylation of downstream target proteins, but also on concomitant reduction of their dephosphorylation. This parallel mechanism is based on the ability of PKA to phosphorylate and activate DARPP-32, a potent inhibitor of protein phosphatase 1 (PP-1). Inhibition of PP-1 suppresses the dephosphorylation of several downstream targets of PKA, thereby amplifying behavioral responses produced by activation of cAMP signaling (Fienberg et al., 1998; Greengard, 2001; Borgkvist and Fisone, 2007).

The sensitization of D1Rs produced by dopamine depletion is reflected by the large increase in DARPP-32 phosphorylation observed in response to the administration of L-DOPA. In rodent models of PD L-DOPA-induced activation of the cAMP/PKA/DARPP-32 cascade has been associated to the emergence of dyskinesia (Picconi et al., 2003; Santini et al., 2007; Lebel et al., 2010). In MPTP lesioned non-human primates, increased phosphorylation of DARPP-32 has been shown to persist for up to 3 months of L-DOPA chronic administration, suggesting that DARPP-32 is involved not only in the development, but also in the maintenance and expression of LID (Santini et al., 2010a).

Genetic inactivation of DARPP-32 has proven to be an effective strategy to reduce experimental LID. Following 6-OHDA lesion and repeated administration of L-DOPA, DARPP-32 knock out mice display significantly less dyskinetic behavior in comparison

to wild type littermates (Santini et al., 2007). Interestingly, LID is also reduced by cell-specific inactivation of DARPP-32 in the MSNs of the direct pathway. In contrast, selective inactivation of DARPP-32 in indirect MSNs does not affect the ability of L-DOPA to induce abnormal involuntary movements (Bateup et al., 2010). Taken together, these studies indicate not only the importance of PKA-induced activation of DARPP-32 in dyskinesia, but also the primary role played in this condition by the D1R-expressing MSNs of the direct pathway.

The abnormal activation of PKA and the concomitant hyperphosphorylation of DARPP-32 observed in experimental models of LID lead to changes in the state of phosphorylation of target effector proteins, which may have profound repercussion on the excitability of striatal MSNs (**Figure 1**). High-frequency stimulation is known to induce long-term potentiation (LTP) at corticostriatal synapses (Calabresi et al., 1992b). Dopamine depletion abolishes LTP, which is rescued by systemic administration of L-DOPA (Centonze et al., 1999; Picconi et al., 2003, 2008). Once established, LTP can be reversed by low frequency stimulation (Picconi et al., 2003, 2008). This phenomenon, called depotentiation, is blocked by inhibition of PP-1 and, most importantly, is absent at the corticostriatal synapses of dyskinetic rats (Picconi et al., 2003, 2008). It has been proposed that depotentiation may prevent the generation of aberrant motor patterns, such as dyskinesia, by erasing non-essential information and normalizing striatal synaptic efficiency (Picconi et al., 2003, 2008; Calabresi et al., 2010). Thus, dyskinesia may be caused by L-DOPA through stimulation of sensitized D1Rs, hyper-activation of PKA, increased phosphorylation of DARPP-32, inhibition of PP-1, and abolishment of corticostriatal depotentiation (Picconi et al., 2003; Calabresi et al., 2010).

One possible mechanism by which inhibition of PP-1 by DARPP-32 may prevent depotentiation involves changes in the state of phosphorylation of the GluR1 subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) glutamate receptor. Dyskinetic behavior correlates with the ability of L-DOPA to increase PKA-dependent phosphorylation of GluR1 at Ser845 (Santini et al., 2007). This effect is strictly dependent on concomitant phosphorylation of DARPP-32, since it is abolished in DARPP-32 knock out mice (Santini et al., 2007). Phosphorylation of GluR1 at Ser845 promotes glutamatergic transmission (Banke et al., 2000; Mangiavacchi and Wolf, 2004) and may participate in the block of depotentiation observed in dyskinetic rats (Picconi et al., 2003). Increased glutamatergic transmission may also be produced by augmented synaptic recruitment of AMPA receptor GluR2/3 subunits, which has been described in the striata of dyskinetic monkeys (Silverdale et al., 2010). The idea of the involvement in dyskinesia of enhanced AMPA receptor transmission is further supported by the observation that, in non-human primates, LID is increased by an AMPA receptor agonist and reduced by an AMPA receptor antagonist (Konitsiotis et al., 2000).

cGMP AND LOSS OF LONG-TERM DEPRESSION IN DYSKINESIA

In the rat, depletion of striatal dopamine results in the loss of corticostriatal long-term depression (LTD). This effect is counteracted by D1R and D2R agonists, exogenous dopamine, or L-DOPA (Calabresi et al., 1992a; Picconi et al., 2008, 2011). Interestingly, recent evidence indicates that in dyskinesia the loss of LTD produced by 6-OHDA persists even after chronic L-DOPA administration (Picconi et al., 2011). This phenomenon, together with the loss of depotentiation (Picconi et al., 2003), is in line with the idea that LID is caused by impaired control of striatal excitatory transmission.

The lack of corticostriatal LTD associated with LID may be due to the decrease in cGMP signaling observed in dyskinetic animals (Giorgi et al., 2008). In the striatum, LTD depends on a group of interneurons, which express nitric oxide synthase (Kawaguchi et al., 1995; Calabresi et al., 1999). Nitric oxide activates a soluble form of guanylyl cyclase highly expressed in MSNs (Ding et al., 2004), leading to increased synthesis of cGMP and activation of cGMP-dependent protein kinase. Notably, cGMP signaling is implicated in LTD, which can be induced pharmacologically with zaprinast and UK-343664, two inhibitors of cGMP phosphodiesterase (the enzyme responsible for the conversion of cGMP to GMP; Calabresi et al., 1999; Picconi et al., 2011). Interestingly, local injection of these drugs in the striatum of dyskinetic rats has been found to rescue LTD and to reduce the dyskinetic response (Picconi et al., 2011). Further studies will be necessary to understand the molecular basis of the persistent loss of LTD associated to dyskinetic behavior.

ENHANCED ERK SIGNALING IN DYSKINESIA

The changes in synaptic plasticity associated to LID, such as loss of depotentiation and LTD are likely to occur also in response to modifications in the activity of signaling pathways involved in the control of gene expression. ERK regulate transcriptional and translational processes (Thomas and Huganir, 2004; Costa-Mattioli et al., 2009) and have been implicated in the induction of striatal LTP (Xie et al., 2009). In neuronal cells, ERK signaling is promoted via Ca^{2+}-dependent activation of the brain specific exchange factor Ras-guanyl nucleotide releasing factor 1 (Ras-GRF1, or $CDC25^{Mm}$; Martegani et al., 1992; Shou et al., 1992). Ras-GRF1 induces the exchange of GDP for GTP on the small G-protein Ras (Farnsworth et al., 1995). A similar effect is produced by the calcium- and diacylglycerol-regulated guanine nucleotide exchange factor II (CalDAG-GEF II), which is highly enriched in striatal MSNs (Toki et al., 2001). Ras-GTP, in turn, activates the protein kinase Raf, leading to the phosphorylation of the mitogen-activated protein kinase/ERK kinase (MEK) and ERK (**Figure 2**).

It has been shown that substances that promote D1R transmission, such as cocaine and amphetamine, phosphorylate ERK via activation of the PKA/DARPP-32 signaling cascade. This effect is involved in the development of the locomotor sensitization produced by repeated administration of these drugs (Valjent et al., 2005). This finding suggests that changes in ERK phosphorylation may also be implicated in LID, which, in experimental models, develops in concomitance with a marked sensitization of the locomotor effect (i.e., turning behavior) produced by L-DOPA (Carey, 1991). Studies performed in 6-OHDA-lesioned rats show that depletion of striatal dopamine confers to a D1R agonist the ability to increase the phosphorylation of ERK (Gerfen et al., 2002). Similarly, L-DOPA, which is inactive when injected in naive or sham-lesioned animals, induces a large increase in ERK

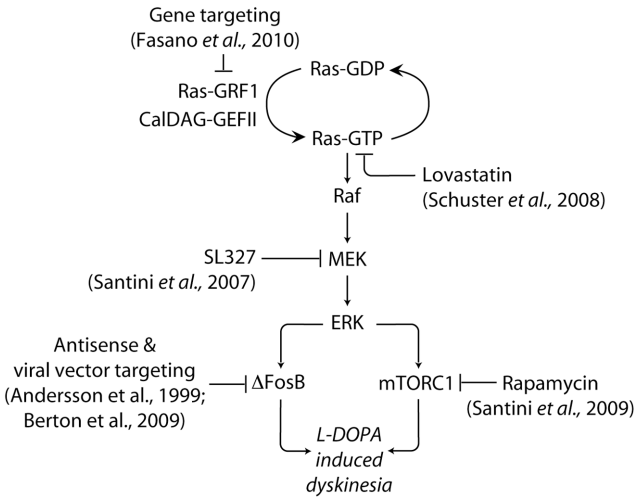

FIGURE 2 | Targeting signaling upstream and downstream of ERK in LID. The abnormal activation of ERK produced by administration of L-DOPA in experimental models of PD is implicated in the emergence of dyskinetic behavior. ERK is activated by Ras-GRF1 and CalDAG-GEF II, which induce the exchange of GDP for GTP on the small G-protein Ras. Ras-GTP activates the protein kinase Raf, leading to the phosphorylation of MEK and ERK. Dyskinesia is attenuated in Ras-GRF1 knock out mice. A similar reduction is produced by inhibition of Ras, or MEK, achieved using lovastatin and SL327, respectively. ERK activation promotes the expression of the transcription factor ΔFosB, which is also implicated in LID. In addition, dyskinesia is associated to ERK-dependent activation of mTORC1, which is likely to accelerate local protein synthesis. Blockade of mTORC1 signaling with rapamycin has been found to reduce LID. See text for abbreviations.

phosphorylation when administered to 6-OHDA-lesioned mice or MPTP intoxicated monkeys (Pavon et al., 2006; Santini et al., 2007, 2009a, 2010a).

The mechanism underlying this change in efficacy is likely to depend on sensitized D1R-mediated transmission and abnormal activation of cAMP signaling. Thus, blockade of PKA prevents the ability of L-DOPA to increase ERK phosphorylation in 6-OHDA-lesioned rats (Lebel et al., 2010). Moreover, in the dorsal striatum, the ability of L-DOPA to activate ERK appears to be attenuated by genetic inactivation of DARPP-32 (Santini et al., 2007). However, this last observation has been recently challenged (Gerfen et al., 2008) and further work will be necessary to assess the involvement of DARPP-32 in L-DOPA-induced ERK phosphorylation, particularly within the dorsolateral striatum.

The existence of a causal link between ERK and dyskinesia was first provided in a mouse model of LID. In particular, it was shown that blockade of ERK phosphorylation with SL327, an inhibitor of MEK, reduced the development of dyskinesia (Santini et al., 2007). Subsequent studies showed that lovastatin, which reduces Ras isoprenylation and activity (Mendola and Backer, 1990; Sebti et al., 1991), attenuates dyskinesia induced by L-DOPA in the 6-OHDA-lesioned rat (Schuster et al., 2008). In the same model, it was found that LID is associated to increased striatal expression of CalDAG-GEF II, which may represent a further mechanism accounting for increased ERK activation (Crittenden et al., 2009; cf. above; **Figure 2**).

The involvement in LID of the Ras–ERK pathway is further supported by recent evidence indicating that dyskinesia is attenuated in Ras-GRF1 knock out mice (Fasano et al., 2010; **Figure 2**). Moreover, in MPTP intoxicated monkeys, viral vector-mediated inhibition of Ras-GRF1 reverses pre-established LID (Fasano et al., 2010). This observation suggests that ERK signaling is involved not only in the development of LID but also in its expression/maintenance. It should be noted, however, that the phosphorylation of ERK produced by L-DOPA in the dopamine-depleted striatum appears to subside over prolonged drug administration. Thus, in dyskinetic MPTP lesioned monkeys, L-DOPA loses its ability to promote ERK phosphorylation following 3 months of chronic treatment (Santini et al., 2010a).

A similar phenomenon has been recently described in mice deficient for the transcription factor Pitx3 (Ding et al., 2011). These animals lack the dopaminergic innervation to the striatum and display akinesia reminiscent of parkinsonism (van den Munckhof et al., 2003; Hwang et al., 2005). In Ptix3 knock out mice, acute L-DOPA increases the number of phospho-ERK-positive MSNs. However, this effect disappears following 7 weeks of daily administration, in concomitance with the appearance of increased phospho-ERK immunoreactivity in cholinergic interneurons (Ding et al., 2011). The progressive reduction in the ability of L-DOPA to activate ERK in striatal MSNs was also observed in 6-OHDA-lesioned mice, further supporting the idea of a partial normalization in ERK signaling occurring over prolonged periods of drug administration (Ding et al., 2011). The increase in phospho-ERK observed in cholinergic neurons is difficult to appreciate using western immunoblotting, since these cells represent only about 2% of striatal neurons (Zhou et al., 2002). Nevertheless, in the striatum, cholinergic interneurons give rise to a particularly dense arborization and to a large number of synaptic contacts (Bolam et al., 1984; Phelps et al., 1985; Contant et al., 1996). Thus, augmented ERK signaling and increased activity in this specific neuronal population may have profound repercussions on striatal MSNs (Ding et al., 2011). In line with this idea, administration of the muscarinic acetylcholine receptor antagonist, dicyclomine, reduces dyskinetic behavior in Ptix3 knock out and 6-OHDA-lesioned mice (Ding et al., 2011).

SEARCHING FOR MECHANISMS OF DYSKINESIA DOWNSTREAM OF ERK

Taken together the studies described above indicate that LID can be counteracted by reducing the activity of the Ras–ERK cascade through pharmacological inhibition of Ras (Schuster et al., 2008) and MEK (Santini et al., 2007). However, this approach is likely to produce negative side-effects, due to the involvement of ERK in basic physiological processes, ranging from synaptic plasticity to cell survival (Orban et al., 1999; Thomas and Huganir, 2004; Kim and Choi, 2010). This limitation can be at least in part resolved by acting on Ras-GRF1, which is specifically expressed in neuronal cells and is not implicated in cell survival (Fasano et al., 2010). Alternatively, negative side-effects can be reduced by narrowing the specificity of intervention through acting downstream of ERK. This approach is particularly interesting, since ERK is involved in the control of a large number of target effector proteins, both in the nucleus and in the cytoplasm (Yoon and Seger, 2006; **Figure 1**).

The mitogen- and stress-activated protein kinase 1 (MSK1) is an important component of the ERK signaling cascade, specifically localized in the nucleus (Deak et al., 1998). LID is accompanied by a large increase in the phosphorylation of MSK1, which occurs selectively in the D1R-expressing neurons of the direct pathway (Santini et al., 2007, 2009a). In MSNs, activation of MSK1 leads to phosphorylation of CREB (Brami-Cherrier et al., 2005), which regulates the expression of genes involved in synaptic plasticity and drug addiction (Carlezon et al., 2005; **Figure 1**). Prolonged administration of L-DOPA has been found to increase CREB phosphorylation in a large proportion of striatal neurons. This effect is mimicked by a D1R agonist and is prevented by inhibition of PKA (Oh et al., 2003), which is known to regulate CREB. In cultured striatal neurons, PKA-dependent phosphorylation of CREB is blocked by inhibition of ERK (Zanassi et al., 2001). Moreover, the ability of cocaine to increase CREB phosphorylation in striatal MSNs is prevented by administration of SL327 (Brami-Cherrier et al., 2005). Therefore, it is likely that the increase in CREB phosphorylation induced in the striatum by repeated administration of L-DOPA is produced by concomitant activation of cAMP/PKA and ERK/MSK1 signaling. The potential implication of altered CREB phosphorylation in LID remains to be fully evaluated, particularly in view of the observation that striatal infusion of antisense oligonucleotide against CREB exacerbates the dyskinetic response to L-DOPA, rather than reducing it (Andersson et al., 2001).

Administration of L-DOPA to 6-OHDA-lesioned rodents results in a large increase in the state of phosphorylation of histone H3 at Ser10 (Santini et al., 2007, 2009a; Darmopil et al., 2009), which is known to depend on activation of MSK1 (Davie, 2003; **Figure 1**). This effect occurs in the MSNs of the direct pathway and correlates with the severity of dyskinetic movements (Santini et al., 2007, 2009a; Darmopil et al., 2009). Increased phosphorylation on Ser10 of histone H3 is thought to mediate transcriptional activation (Nowak and Corces, 2004) and may participate in changes in gene expression associated to LID.

ERK-DEPENDENT CHANGES IN GENE EXPRESSION ASSOCIATED TO DYSKINESIA

Evidence obtained in rodent and non-human primate models indicates that LID is accompanied by increased levels of stable isoforms of ΔFosB (Andersson et al., 1999; Pavon et al., 2006; Berton et al., 2009; Darmopil et al., 2009; Fasano et al., 2010), a truncated splice variant of the immediate early gene *fosB* (Nestler et al., 2001; **Figures 1** and **2**). Importantly, increased ΔFosB has also been observed in postmortem striatal samples from parkinsonian patients treated with L-DOPA (Tekumalla et al., 2001). ΔFosB acts as a transcriptional activator by forming heterodimers with JunD, which bind to the activator protein-1 site of several late response genes. Enhanced expression of ΔFosB has been implicated in the long-term effects produced by substances of abuse, including dopaminergic drugs such as cocaine (Nestler et al., 2001).

The increase in ΔFosB associated to LID is restricted to the D1R-expressing MSNs of the direct pathway (Andersson et al., 1999; Pavon et al., 2006) and is mediated via activation of the D1R/cAMP cascade. Thus, genetic inactivation of D1Rs, or striatal infusion of Rp-cAMPS prevent the ability of L-DOPA to increase ΔFosB (Darmopil et al., 2009; Lebel et al., 2010). The accumulation of ΔFosB requires also PKA-catalyzed phosphorylation of DARPP-32 (Zachariou et al., 2006), which is strongly induced by administration of L-DOPA (Santini et al., 2007). ERK signaling is also involved in the regulation of ΔFosB, since inactivation of Ras-GRF1 (Fasano et al., 2010), or pharmacological inhibition of Ras (Schuster et al., 2008), abolish the increase in ΔFosB expression induced by L-DOPA.

Several lines of evidence indicate that the up-regulation of ΔFosB produced by chronic administration of L-DOPA participates in the development of dyskinesia. Studies performed in 6-OHDA-lesioned rats showed that LID is reduced by striatal injection of a *fosB* antisense oligonucleotide (Andersson et al., 1999; **Figure 2**). In the same model, it has been recently shown that viral vector-induced overexpression of ΔFosB enhances the ability of L-DOPA to induce dyskinetic behavior (Cao et al., 2010). Furthermore, experiments in MPTP lesioned macaques showed that overexpression of ΔJunD, a truncated variant of JunD lacking a transactivation domain and acting as a dominant negative inhibitor of ΔFosB, reduces dyskinesia (Berton et al., 2009; **Figure 2**).

Previous studies showed that repeated administration of a D1R agonist to 6-OHDA-lesioned rats increases the expression of mRNA coding for the opioid peptide prodynorphin in the MSNs of the direct pathway (Gerfen et al., 1990). A similar regulation was later found to correlate with dyskinesia and to depend on L-DOPA-induced expression of ΔFosB (Andersson et al., 1999; **Figure 1**). Although increased prodynorphin is regarded as a very robust marker of LID, a clear understanding of the role played by enhanced opioid transmission in dyskinesia is complicated by contrasting data obtained using opioid receptor antagonists (Samadi et al., 2006). Further studies will be necessary to fully characterize the significance of this and other effects dependent on ΔFosB for the development and expression of LID.

Another immediate early gene whose expression is increased in dyskinesia is that coding for the transcription factor Zif268 (or NGFI-A/Krox24/Egr1). Acute administration of L-DOPA to 6-OHDA-lesioned rats increases *zif268* mRNA in both striatopallidal and striatonigral MSNs. Interestingly, repeated L-DOPA administration leading to dyskinesia normalizes the levels of *zif268* mRNA in striatopallidal neurons, but not in striatonigral neurons (Carta et al., 2005). The lack of normalization of *zif268* expression in the MSNs of the direct pathway may be due to the persistent activation of ERK observed in these cells in association with LID (Darmopil et al., 2009; Santini et al., 2009a). Indeed, ERK is required for cocaine-induced increase in *zif268* expression (Valjent et al., 2006). Interestingly, Zif268 is involved in the sensitized motor response produced by repeated administration of cocaine (Valjent et al., 2006), suggesting that activation of the ERK/Zif268 cascade may represent a common mechanism implicated in abnormal motor behaviors (**Figure 1**).

In the hippocampus, Zif268 is required for the late phase of LTP (Jones et al., 2001). This regulation is thought to depend on the ability of Zif268 to induce Arc (or Arg3.1; Li et al., 2005), another immediate early gene involved in multiple forms of neuronal plasticity, including LTP and LTD (Bramham et al., 2008). Interestingly, increased expression of Arc has been found in the striata of dyskinetic rats, specifically at the level of the MSNs of

the direct pathway (Sgambato-Faure et al., 2005). These observations raise the possibility that activation of the ERK/Zif268/Arc signaling pathway may participate in the plastic changes associated to LID. In particular, abnormal increase of Zif268 and Arc may promote or exacerbate LTP, leading to the loss of corticostriatal depotentiation associated to dyskinesia (Picconi et al., 2003; **Figure 1**).

ERK-DEPENDENT REGULATION OF MAMMALIAN TARGET OF RAPAMYCIN COMPLEX 1

The involvement of ERK in LID may depend not only on the regulation of transcription factors (i.e., CREB, ΔFosB, and Zif268), but also on the modulation of local protein synthesis in the cytoplasm. In this regard, the mammalian target of rapamycin complex 1 (mTORC1), a key regulator of mRNA translation (Costa-Mattioli et al., 2009), is emerging as a particularly interesting subject of study (Santini et al., 2010b; **Figure 1**).

ERK has been proposed to activate mTORC1 through several mechanisms. For instance, ERK may promote the activation of the small GTPase, Ras homolog enriched in brain (Rheb), which stimulates mTORC1 (Long et al., 2005; Ma et al., 2005). In addition, ERK can phosphorylate and activate the regulatory associated protein of mTOR (or Raptor), an essential component of mTORC1, thereby promoting mTORC1-mediated signaling (Carriere et al., 2008).

Studies performed in the hippocampus showed that activated mTORC1 participates to the maintenance of LTP by promoting local protein synthesis immediately after synaptic stimulation (Tsokas et al., 2005). This effect, which requires activation of ERK (Tsokas et al., 2007), may be implicated in the maladaptive processes associated with LID, which affect the functioning of corticostriatal synapses (see above; Picconi et al., 2003, 2011; Calabresi et al., 2010). In line with this possibility, it has been found that administration of L-DOPA to 6-OHDA-lesioned mice promotes the phosphorylation of the p70 ribosomal S6 kinases (S6Ks) and the initiation factor 4E (eIF4E)-binding protein (4E-BP; Santini et al., 2009b), two major targets of mTORC1 (Thomas et al., 1979; Gingras et al., 2001; Ruvinsky and Meyuhas, 2006). These effects, which lead to activation of the eIF4E and to the phosphorylation of the ribosomal protein S6 (rpS6), are thought to accelerate protein synthesis (Richter and Sonenberg, 2005; Ruvinsky and Meyuhas, 2006; Roux et al., 2007).

The increase in mTORC1 signaling produced by L-DOPA occurs selectively in the MSNs of the direct pathway and requires concomitant activation of ERK signaling (Santini et al., 2009b). Blockade of mTORC1 does not interfere with the ability of L-DOPA to counteract the akinetic effect of the 6-OHDA lesion, but it diminishes the development of LID. Thus, combined administration of L-DOPA and rapamycin, a selective allosteric inhibitor of mTORC1 (Oshiro et al., 2004), leads to a substantial reduction in the emergence of abnormal involuntary movements in dopamine-depleted mice (Santini et al., 2009b; **Figure 2**). These results suggest that enhanced mRNA translation, leading to abnormal protein synthesis may participate in the development of LID. In this regard, it will be important to identify downstream effectors specifically affected by abnormal mTORC1 signaling (Klann, 2009; Santini et al., 2009b, 2010b).

CONCLUSION

The study of the molecular mechanisms at the basis of LID has unveiled a number of abnormalities in signal transduction associated to the development and manifestation of this condition (cf. **Figures 1** and **2**). Most importantly, it has been shown that drugs targeting specific intracellular components of the signaling machinery in striatal MSNs efficiently counteract the emergence of LID. These drugs include inhibitors of the Ras–ERK and mTORC1 cascades, such as SL327, lovastatin, and rapamycin (**Figure 2**), as well as inhibitors of the cGMP phosphodiesterase, such as zaprinast and UK-343664. Although some of these substances (e.g., lovastatin and rapamycin) have been tested in clinical settings, their use for the treatment of dyskinesia may present problems related to long-term side-effects at both central and peripheral level. This indicates the importance of testing additional substances interacting with signaling changes potentially implicated in LID and occurring downstream of the ERK and mTORC1 cascades.

This review focused on abnormal signaling at the level of the D1R-expressing MSNs of the direct striatonigral pathway. Whereas acting on these neurons seems to produce beneficial effects against dyskinetic behavior, other neuronal populations may represent equally feasible targets. For instance, pharmacological and genetic interventions aimed at modifying transmission in the D2R-expressing MSNs of the indirect pathway have been shown to produce anti-dyskinetic effects (Schwarzschild et al., 2006; Gold et al., 2007; Schuster et al., 2009). This is not surprising, since both direct and indirect MSNs act in large part by controlling the same set of structures (i.e., substantia nigra pars reticulata and internal segment of the globus pallidus), ultimately involved in the control exerted by the basal ganglia on motor function. Recent work indicates that other groups of striatal neurons are also involved in LID. In particular, it has been shown that dyskinesia may depend on changes in the activity of cholinergic interneurons, as well as of interneurons expressing nitric oxide synthase. One important question in the study of the mechanisms of dyskinesia will be to identify signaling abnormalities associated with LID and occurring at the level of these distinct neuronal populations, to understand their impact on basal ganglia transmission and to assess their contribution to dyskinetic behavior.

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Dysregulated mTORC1-dependent translational control: from brain disorders to psychoactive drugs

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In the last decade, a plethora of studies utilizing pharmacological, biochemical, and genetic approaches have shown that precise translational control is required for long-lasting synaptic plasticity and the formation of long-term memory. Moreover, more recent studies indicate that alterations in translational control are a common pathophysiological feature of human neurological disorders, including developmental disorders, neuropsychiatric disorders, and neurodegenerative diseases. Finally, translational control mechanisms are susceptible to modification by psychoactive drugs. Taken together, these findings point to a central role for translational control in the regulation of synaptic function and behavior.

Keywords: protein synthesis, translation initiation, mTORC1 signaling, S6K1, eIF4E, developmental disorders, neurodegenerative diseases, psychoactive drugs

INTRODUCTION

The critical importance of *de novo* protein synthesis as a molecular mechanism involved in the process of consolidating storage of information in the brain has been shown in numerous experimental systems using a variety of pharmacological and genetic approaches (Neves et al., 2008). For instance, one of the first behavioral studies performed in rodents treated with the protein synthesis inhibitor puromycin demonstrated that loss of memory for avoidance discrimination learning was caused by blockade of protein synthesis (Flexner et al., 1963).

Information is encoded and stored in the brain at the level of individual synapses, which represent the "cellular unit of memories" (Hoeffer and Klann, 2010). Synapses are "plastic" in that their physiological efficacy (strength) changes and outlasts the events that trigger them. These persistent, activity-dependent changes in synaptic strength are triggered by *de novo* protein synthesis (Klann and Sweatt, 2008). Evidence indicating that protein synthesis can occur at specific synaptic sites comes from electron microscopy studies that demonstrated the presence of polyribosomes in dendrites of dentate granule neurons (Steward and Levy, 1982; Steward and Schuman, 2001). Accordingly, electrical stimulation of CA1 pyramidal cell dendrites led to the rapid incorporation of radiolabeled amino acids into newly synthesized proteins (Feig and Lipton, 1993). The functional role of dendritic protein synthesis then was demonstrated in hippocampal slices where newly synthesized proteins were required to enhance synaptic strength induced by brain-derived neurotrophic factor (BDNF; Kang and Schuman, 1996). In addition, dendritic protein synthesis is necessary for long-lasting decreases in synaptic strength induced by activation of group I metabotropic glutamate receptors (Huber et al., 2000). These findings indicate that protein synthesis is triggered at synaptic locations and is required for several forms of synaptic plasticity, which in turn is thought to be essential for higher brain functions, including memory formation.

Biochemical signaling mechanisms coordinating the process of neuronal protein synthesis are highly regulated and can be separated into three general phases: initiation, elongation and termination. The vast majority of the known translation regulation occurs at the level of the initiation phase and involves the coordinate activities of numerous molecular factors (Costa-Mattioli et al., 2009a; Richter and Klann, 2009; Sossin and Lacaille, 2010). Critical to the regulation of translation initiation is the activity of mammalian target of rapamycin (mTOR), a kinase ubiquitously expressed in eukaryotes (Hoeffer and Klann, 2010). In this review, we describe molecular mechanisms that control translation initiation and discuss examples of how perturbations of this regulation are a common pathophysiological feature of human neurological disorders.

mTOR SIGNALING PATHWAY AND TRANSLATION INITIATION

Mammalian target of rapamycin function is regulated by the activity state of several neuronal surface receptors and channels (i.e., TrkB, NMDAR, AMPA, D1R, D2R, mGluRs) and by diverse signaling cascades activated by these receptors, including PDK1, PI3K, Akt, and TSC1/2 (Averous and Proud, 2006; Wang and Proud, 2006). mTOR is a serine–threonine kinase and exists as one component of protein complexes termed mTORCs (**Figure 1**) that include numerous factors regulating mTOR signaling and controlling the specificity for its diverse effectors targets, as well as directing its subcellular localization (Cutler et al., 1999; Martin and Blenis, 2002).

mTORCs are defined primarily by the key scaffold proteins interacting with mTOR, Raptor, and Rictor. mTOR, when found in a complex with Raptor is known as mTORC1, whereas mTOR bound to Rictor is known as mTORC2 (Beretta et al., 1996; Kim et al., 2002; Sarbassov et al., 2004). These two complexes are thought to participate in the regulation of diverse signaling pathways that mediate different neuronal functions. Indeed, mTORC1 is primarily involved in the regulation of cap-dependent

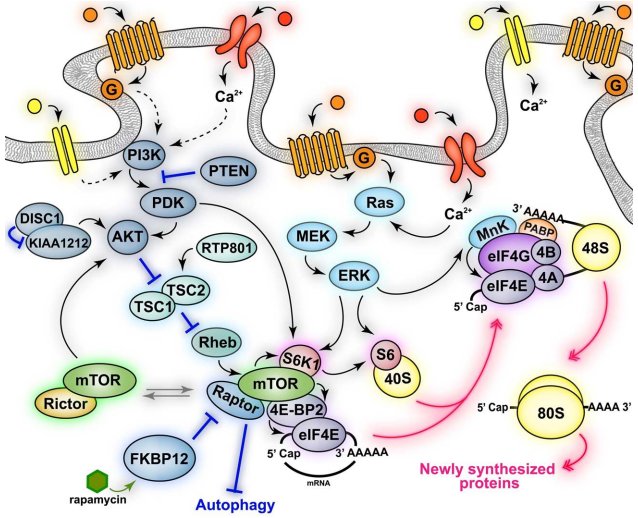

FIGURE 1 | Schematic of mTOR signaling pathways. Activation of neuronal receptors and channels (mGluRs, NMDARs, TrkB, D1Rs, and D2Rs) leads to activation of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 activation increases some neuronal processes (protein synthesis) while inhibiting others (autophagy). mTORC1 regulates the activity of downstream effectors involved in translation (S6K1, 4E-BP2), some of which are also directly phosphorylated via convergent activation of the MAPK signaling pathway (S6K1, MnK, S6). mTORC1-dependent phosphorylation of 4E-BP2 results in the association of eIF4E with eIF4G and the formation of the active eIF4F (eIF4E–eIF4A–eIF4G) complex. eIF4F recruits eIF4B and MnK and promotes the binding of mRNAs to the 43S pre-initiation complex to form the 48S initiation complex. The eIF4F complex and the poly(A) tail act synergistically together with MnK-dependent phosphorylation of eIF4E to stimulate cap-dependent translation initiation. The signaling pathways that activate mTORC2 and its downstream effectors, as well as its physiological functions are currently unknown. Black solid arrows indicate direct phosphorylation/activation, pink double arrows represent molecular association/dissociation and blue lines indicate inhibition.

translation initiation, whereas mTORC2 is implicated in structural modifications such as cytoskeletal rearrangements (Jacinto and Hall, 2003; Hay and Sonenberg, 2004). Furthermore, these two complexes have different sensitivity to rapamycin, which does not inhibit mTOR kinase activity *per se*, but rather interferes with the binding of mTOR to Raptor, thereby disrupting mTORC1 and the phosphorylation of its substrates (**Figure 1**). Thus, mTORC1, but not mTORC2, is inhibited by rapamycin (Beretta et al., 1996; Kim et al., 2002), although prolonged treatment with rapamycin in cultured cells may also indirectly inhibit mTORC2 (Sarbassov et al., 2006).

Critical downstream targets of mTORC1 that are involved in translation include the p70 ribosomal S6 kinases 1 and 2 (S6K1 and S6K2) and the eIF4E-binding proteins (4E-BP; Klann and Dever, 2004). S6K1 is involved in the phosphorylation of the ribosomal protein S6 (**Figure 1**), an essential component of the 40S ribosomal subunit, and regulates its synthesis. The role of S6 in the regulation of translation is not well understood. S6 is located in close proximity to mRNA- and tRNA-binding sites, and genetic deletion of S6 impairs ribosome biogenesis and cell proliferation (Volarevic et al., 2000). S6K1 also mediates the phosphorylation of initiation factor 4B (eIF4B), which increases the catalytic activity of eIF4A (Rogers et al., 2001b; Raught et al., 2004), an RNA helicase critical for the translation of mRNA with complex 5′UTR secondary structures (Rogers et al., 1999, 2001a). Furthermore, S6K1 is also involved in translation elongation via phosphorylation of elongation factor 2 (eEF2) kinase. Phosphorylation of eEF2 kinase by S6K1 enhances the elongation rate and presumably protein synthesis by inhibiting its kinase activity, thereby decreasing the phosphorylation of eEF2 (Wang et al., 2001).

4E-BPs, which will be referred to from this point on as 4E-BP2 (**Figure 1**) because it is the predominant isoform in the mammalian brain (Banko et al., 2005), bind to the cap-binding protein eIF4E and repress its interaction with eIF4G and the other factors involved in formation of the initiation complex (Pause et al., 1994; Haghighat et al., 1995; Fletcher et al., 1998; Poulin et al., 1998). Thus, 4E-BP2 blocks eIF4F formation and inhibits protein synthesis by competing with eIF4G for the binding of eIF4E (**Figure 1**). The repressor activity of 4E-BP2 is regulated by sequential phosphorylation of its numerous phosphorylation sites (Bidinosti et al., 2010). The various 4E-BP2 residues are phosphorylated by diverse kinases in a sequential manner, with the last phosphorylation event occurring by mTORC1 (Gingras et al., 1999, 2001). Thus, mTORC1-dependent phosphorylation of 4E-BP2 releases eIF4E, which in turn associates with eIF4G. The binding of eIF4E to eIF4G and eIF4A forms the eIF4F initiation complex, which also recruits eIF4B (**Figure 1**). Formation of eIF4F complex is the crucial molecular event that triggers translation initiation. The formation of the eIF4F complex leads to the circularization of the mRNA through the interaction between cap-eIF4E, eIF4G, and poly A-binding proteins (PABP; **Figure 1**), which recruits ribosomes and promotes the unwinding of the secondary structure of 5′UTR mRNA, thereby facilitating the recognition of the start codon (Gingras et al., 1999; Klann and Dever, 2004). The eIF4F complex is further regulated through phosphorylation of some of its factors, such as eIF4G and eIF4E, which are phosphorylated by mTORC1 and Mnk1/2, respectively (**Figure 1**). Currently, the functional effect of the phosphorylation of eIF4G is not clear, whereas phospho-eIF4E is thought to facilitate translation initiation (Gingras et al., 1999; Pyronnet et al., 1999; Waskiewicz et al., 1999; Raught et al., 2000).

ABERRANT mTORC1 ACTIVITY IS A COMMON MOLECULAR PATHWAY FOR DISEASES OF CNS

Initially, the role of mTORC1 signaling in the physiology of the nervous system was studied to understand the causal relationship between mTOR-dependent translation and either brain synaptic plasticity or memory formation (Costa-Mattioli et al., 2009a; Banko and Klann, 2008; Costa-Mattioli and Sonenberg, 2008). The availability of specific pharmacological inhibitors of upstream kinases, together with genetically modified mice that have deletions in either upstream activators or downstream effectors have greatly accelerated our understanding about the role of mTORC1 in nervous system function. In general, these studies have revealed that mTORC1 critically modulates protein synthesis-dependent synaptic plasticity and memory (Tang et al., 2002; Cammalleri et al., 2003; Tischmeyer et al., 2003; Hou and Klann, 2004; Tsokas et al., 2005; Dash et al., 2006; Gelinas et al., 2007; Antion et al.,

2008a,b; Hoeffer et al., 2011). In addition, deletion of negative regulators of mTORC1 results in aberrant synaptic plasticity and altered memory function (Banko et al., 2005, 2006, 2007; Costa-Mattioli et al., 2009b; Banko and Klann, 2008; Swiech et al., 2008; Hoeffer and Klann, 2010). Thus, unbalanced mTORC1 signaling that perturbs the precise regulation of protein synthesis appears to be detrimental and have pathological consequences for nervous system function. These findings are important in light of the increasing amount of evidence linking dysregulated mTOR signaling as a common molecular mechanism involved in the etiology of several neurological disorders. In the next few sections we will discuss recent studies implicating mTORC1-dependent protein synthesis in disorders of the nervous system.

NEURODEVELOPMENTAL DISORDERS

Neurodevelopmental disorders are defined as diseases caused by abnormal development of the brain during the first two decades of life. These disorders have a strong and complex genetic basis with diverse expression and thus, their clinical diagnosis is complicated by a broad array of symptoms (Grice and Buxbaum, 2006; Persico and Bourgeron, 2006; Levitt and Campbell, 2009). However, it is possible to define a subgroup of these disorders that are characterized by single-gene mutations and behavioral impairments similar to those reported in autistic patients. Autism is diagnosed based on the appearance of behavioral abnormalities that include deficits in social interaction skills, impaired ability to communicate, and ritualistic-like repetitive behaviors (Hill and Frith, 2003). A common molecular abnormality in several of these autism-like disorders is loss-of-function mutations and/or deletion of genes that encode proteins that normally repress mTORC1 signaling pathway. These observations led to the hypothesis that dysregulation of protein synthesis may be the cause of altered synaptic development and plasticity, resulting in autistic-like phenotypes in humans (Kelleher and Bear, 2008; Bourgeron, 2009; Hoeffer and Klann, 2010).

Fragile X syndrome

Fragile X syndrome (FXS) is an X-linked form of metal impairment caused by transcriptional silencing of the *FMR1* gene. The fragile X mental retardation protein (FMRP), encoded by the *FMR1* gene, is a RNA-binding protein that represses the translation of specific mRNAs, such as MAP1B Arc/Arg3.1, αCaMKII, PSD-95, SAPAP3 (Todd et al., 2003; Zalfa et al., 2003; Hou et al., 2006; Muddashetty et al., 2007; Narayanan et al., 2008). Consistent with this idea, ablation of FMRP has been shown to increase the levels of the proteins encoded by these specific mRNAs. Thus, the etiology of FXS, resulting from the absence of functional FMRP, is an exaggerated synthesis of specific proteins. However, it also has been shown that general protein synthesis is exaggerated in FXS model mice (Qin et al., 2005; Dölen et al., 2007; Osterweil et al., 2010). It has been proposed that the molecular mechanism mediating FMRP-dependent repression of translation initiation is inhibition of interactions of eIF4E and eIF4G (**Figure 2**). Indeed, FMRP interacts with a non-canonical 4E-BP, named cytoplasmic FMRP Interacting protein 1 (CYFIP1; Napoli et al., 2008). In addition, it has been demonstrated that FXS model mice have exaggerated mTORC1 signaling in the hippocampus resulting from increased

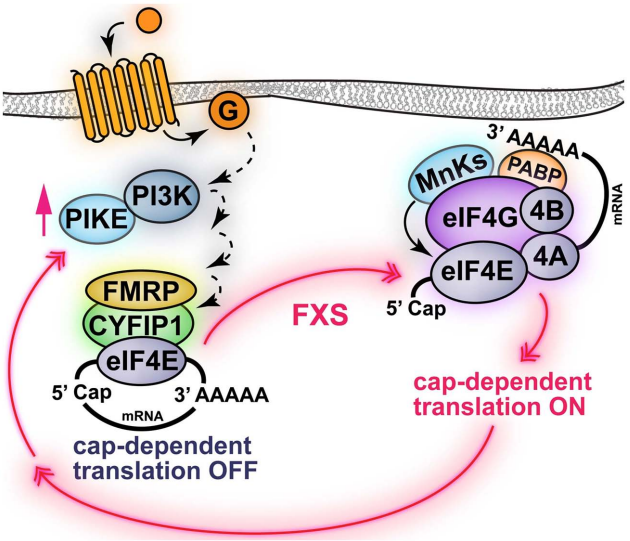

FIGURE 2 | Schematic of FMRP regulation of cap-dependent translation initiation. FMRP represses the translation of specific mRNAs via interaction with a 4E-BP-like protein termed CYFIP1, which also binds eIF4E. One of the mRNAs regulated by FMRP is encoding for PIKE, which enhances PI3K signaling. In fragile X syndrome (FXS), the absence of functional FMRP results in the abnormal translation of PIKE and other mRNAs. Indeed, in FXS model mice PI3K/mTORC1 signaling is enhanced. Note that activation of surface receptors (i.e., group I mGluRs) promotes PI3K signaling via PIKE and activation of mTORC1-dependent protein synthesis. Activation of mTORC1 may also induce translation of FMRP-dependent mRNAs via an unknown mechanism. Black solid arrows indicate direct phosphorylation/activation, dashed arrows represent events mediated by unknown molecular effectors.

expression of PIKE. Indeed, PIKE mRNAs are repressed by FMRP and encode for a GTPase that enhances PI3K activity. Thus, PIKE is abnormally synthesized in absence of FMRP resulting in enhanced PI3K–Akt–mTORC1 signaling (**Figure 2**). These observations are consistent with the idea that aberrant cap-dependent translation is involved in FXS (Gross et al., 2010; Sharma et al., 2010). The molecular abnormalities of FXS model mice are accompanied by several behavioral deficits, which include impairments in hippocampus-dependent learning and memory in the Morris water maze and terrestrial radial arm maze paradigms (Kooy et al., 1996; D'Hooge et al., 1997; Mineur et al., 2002). In addition, FXS model mice showed increased latency in finding the new platform position in the reversal phase of the Morris water maze test, where the position of the hidden platform is suddenly changed (D'Hooge et al., 1997). This suggests that FXS model mice not only have learning and memory deficits, but also behavioral inflexibility in switching to a new searching strategy. FXS model mice also exhibit impaired contextual fear conditioning (Van Dam et al., 2000), altered sensorimotor integration (Chen and Toth, 2001; Nielsen et al., 2002), and increased susceptibility to audiogenic epileptic seizures (Musumeci et al., 2000; Chen and Toth, 2001).

Tuberous sclerosis complex

Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutations in either hamartin (TSC1) or tuberin

(TSC2), which negatively regulate the mTORC1 signaling pathway, and is clinically characterized by a high prevalence of autism and cognitive impairments (Wiznitzer, 2004; Kwiatkowski and Manning, 2005; Wullschleger et al., 2006). TSC1 is a chaperone protein and TSC2 is a GTPase-activating protein (GAP) with catalytic activity directed toward the small G protein Rheb (Inoki et al., 2003). Loss-of-function mutations affecting TSC1/TSC2 result in the abolishment of the GAP activity of TSC2 (Garami et al., 2003). mTORC1 activation is typically regulated by TSC2 (**Figure 1**), which mediates the binding of GTP–Rheb (Ras homologs enriched in the brain) to the mTORC1 kinase domain (Zhang et al., 2003). Thus, TSC1/TSC2 mutations result in the exaggerated activation of mTORC1 signaling pathway (Manning and Cantley, 2003a,b).

Mouse models utilized to study TSC have been engineered with heterozygous mutations in the genes for either TSC1 or TSC2. TCS model mice have been observed to display several hippocampus-dependent learning and memory deficits, including impaired spatial learning and memory, and abnormalities in contextual fear conditioning (Goorden et al., 2007; Ehninger et al., 2008b) reminiscent of behavioral phenotypes displayed by FXS model mice. In addition, TSC1 heterozygous knockout mice and dominant/negative TSC2 mutant mice also displayed impaired social behaviors (Goorden et al., 2007; Chevere-Torres et al., 2012), whereas TSC2 heterozygous knockout mouse pups exhibited abnormalities in social communication as demonstrated by changes in ultrasonic vocalizations following maternal separation (Young et al., 2010). Together, these observations suggest that mutation of only one of the two alleles encoding for the TSC proteins is sufficient to induce cognitive deficits. Importantly, the mTORC1 inhibitor rapamycin has been shown to be effective in ameliorating learning and memory deficits in TSC2 heterozygous knockout mice (Ehninger et al., 2008b), suggesting that uncontrolled mTORC1 signaling is a core molecular mechanism involved in the behavioral abnormalities. Moreover, the TSC model mice display behavioral abnormalities in the absence of neuropathological features such as brain tumors (tubers) and abnormalities in neuronal spine density, which were assumed to be responsible for the cognitive deficits displayed by TSC patients (Goorden et al., 2007; Ehninger et al., 2008a; Chevere-Torres et al., 2012). These findings imply that the pathological brain abnormalities in TSC are not necessary for the occurrence of behavioral deficits, suggesting that their appearance results from the activity of other molecular factors.

Mutation of phosphatase and tensin homolog on chromosome 10

Phosphatase and tensin homolog on chromosome 10 (PTEN) is a tumor suppressor gene that is mutated in many human cancers (Ali et al., 1999) as well as in brain disorders such as autism, mental impairment, and macrocephaly (Zori et al., 1998; Goffin et al., 2001; Waite and Eng, 2002; Butler et al., 2005). PTEN has lipid phosphatase activity and antagonizes the phosphatidylinositol 3-kinase (PI3K)-dependent signaling (**Figure 1**) by dephosphorylating the lipid targets of PI3K (Maehama and Dixon, 1999). Thus, loss of PTEN function in neurons results in hyperactivation of mTORC1, which is a downstream target of the PI3K/Akt signaling pathway (Kwon et al., 2006a). Mice with genetic deletion of PTEN restricted to a subset of differentiated hippocampal and cortical neurons were shown to exhibit behavioral impairments consistent with clinically relevant autistic behaviors. For example, the conditional PTEN mutant mice exhibited abnormalities in social behavior accompanied by deficits in pre-pulse inhibition, anxiety-like behaviors, and seizures (Kwon et al., 2006a,b). The behavioral abnormalities of PTEN mutant mice were observed in the absence of dramatic brain pathologies, which is similar to what was observed in TSC heterozygous knockout mice (Kwon et al., 2006a).

Phosphatase and tensin homolog on chromosome 10 mutant mice have been shown to develop macrocephaly as well as abnormalities in dendritic arborization and axonal growth (Kwon et al., 2006a). Importantly, pharmacological attenuation of mTORC1 activity with rapamycin reduced the impairments in social interactions and the duration and frequency of spontaneous seizures as well as neuronal hypertrophy responsible for the macrocephaly. This suggests that exaggerated mTORC1 signaling pathway is responsible for the behavioral abnormalities observed in PTEN mutant mice (Zhou et al., 2009). Thus, these findings are consistent with the notion that upregulated mTORC1 signaling results in behavioral abnormalities in both TSC model mice and PTEN mutant mice.

Deletion of FKBP12 gene

Genetic studies demonstrate that chromosomal deletions immediately flanking the gene encoding for FK506-binding protein 12 *(FKBP12)* are present in autistic patients (Sebat et al., 2007). FKBP12 is an immunophilin that binds rapamycin and regulates the phosphorylation of mTORC1 substrates by blocking the formation of the mTORC1 complex (Jacinto and Hall, 2003). Thus, genetic deletion of FKBP12 should result in exaggerated mTORC1 activity (**Figure 1**). The hypothesis that increased mTORC1 signaling is involved in the onset of autism-like syndromes has been tested with conditional knockout mice in which the *FKBP12* gene was removed in the forebrain at approximately 3 weeks of age (Hoeffer et al., 2008). Deletion of FKBP12 was associated with increased activity of mTORC1 as shown by an augmented interaction between mTOR and Raptor and enhanced phopshorylation of the mTORC1 target S6K1 (Hoeffer et al., 2008). The changes in mTORC1 signaling in the FKBP12 conditional knockout mice were accompanied by cognitive abnormalities, including enhanced associative contextual fear conditioning, preference for the familiar object in the novel object recognition test, and behavioral inflexibility in several reversal learning paradigms (Hoeffer et al., 2008). These findings suggest that exaggerated mTORC1 activation leads to multiple behavioral abnormalities, including increased perseveration. Furthermore, these results imply that autistic behaviors in syndromes with upregulated mTORC1 signaling are not entirely predetermined during development because dysregulation of mTORC1 signaling in FKBP12 conditional knockout mice occurs postnatally.

NEUROPSYCHIATRIC DISORDERS

Depression

Depression is a neuropsychiatric disorder characterized by anhedonia, irritability, depressed mood, difficulties in concentrating, and abnormalities in appetite and sleep (Krishnan and Nestler,

2008). Complications in the current pharmacotherapy for depression are related mainly to the long duration of time required by the treatment to produce a therapeutic response and the abundance of treatment-resistant patients (Clark et al., 2009). Clinical studies revealed that low doses of ketamine, a non-selective NMDA receptor (NMDAR) antagonist, produces a rapid and sustained antidepressant effects that are also observed in patients with treatment-resistant depression (Berman et al., 2000; Zarate et al., 2006; Price et al., 2009). Thus, NMDARs may play an important role not only in the pathophysiology of depression, but also as a novel molecular target for the mechanism of action of antidepressant drugs. However, the molecular connection between antagonism of NMDARs and antidepressant behavioral effects is unknown. Recently, two independent studies demonstrated that administration of a non-anesthetic dose of ketamine produced antidepressant-like behavior in rodents but the molecular mechanisms responsible for this effect appear to be different.

Nanxin et al. (2010) showed that ketamine transiently activates mTORC1 signaling in the prefrontal cortex (PFC) of rats. This treatment results in mTORC1-dependent synthesis of new pre- and postsynaptic proteins, such as Arc, synapsin, PSD-95, and GluR1, that are required for plastic changes at new spines and synapses. Accordingly, the enhanced mTORC1-dependent synthesis of synapse-associated proteins was accompanied by a rapid increase in spine density and increased excitatory postsynaptic currents (EPSCs) in layer V pyramidal neurons in the PFC (Nanxin et al., 2010). Thus, acute ketamine administration results in enhanced mTORC1-dependent protein synthesis, which leads to long-term changes in PFC neurons, such as an increase in synapses and spine formation, and enhanced synaptic strengthening. Importantly, intracerebroventricular (ICV) infusion of rapamycin blocked all the physiological effects induced by ketamine, which strongly suggests that the formation of new spines and increases in synaptic strength and antidepressant-like behaviors were functional consequences of activation of mTORC1. Furthermore, "depressed rats" exhibit reduced levels of GluR1, PSD-95, and synapsin I in the PFC, and a single dose of ketamine normalized the levels of these proteins in an mTORC1-dependent manner (Nanxin et al., 2010). These results indicate that depression may be the pathophysiological consequence of altered spine remodeling and synaptic plasticity in the PFC.

In contrast, Autry et al. (2011) demonstrated that the antidepressant effects of ketamine are mediated by the synthesis of BDNF in the hippocampus. Indeed, increased BDNF expression was blocked by anisomycin, which is a general inhibitor of protein synthesis. Reduction of BDNF levels in the hippocampus also was accompanied by blockade of antidepressant-like behavior. Furthermore, BDNF and neurotrophic tyrosine kinase receptor type 2 (TrkB receptor) null mice were resistant to the antidepressant effects of ketamine, strongly suggesting that newly translated BDNF mediates the positive, antidepressant effects of NMDA receptor blockade. The authors also demonstrated that acute blockade of NMDA receptor decreased phosphorylation of elongation factor 2 (eEF2), which normally promotes the ribosomal translocation of the nascent polypeptide chain. Phosphorylation of eEF2 inhibits its catalytic activity and blocks ribosomal translocation, thereby halting ribosomes and blocking protein synthesis. Mice pharmacologically treated with inhibitors of eEF2 phosphorylation showed antidepressant-like behavior accompanied by increased BDNF levels in the hippocampus (Autry et al., 2011). These findings suggest that reduction of eEF2 phosphorylation is sufficient to disinhibit protein synthesis, stimulate translation of BDNF, and promote antidepressant-like behaviors. Thus, drugs targeting eEF2 kinase activity are a potential novel pharmacological treatment for depression.

It appears that antidepressant drugs share the ability of increase protein synthesis. Indeed, this is a common molecular mechanism of drugs antagonizing NMDA receptors (ketamine; Nanxin et al., 2010; Autry et al., 2011) as well as inhibiting the reuptake of serotonin (fluoxetine; Dagestad et al., 2006). Thus, one could reasonably conclude that dysregulation of translational control is a molecular mechanism involved in the onset of depressive syndromes.

Schizophrenia

The involvement of mTORC1 signaling in schizophrenia has been suggested by genetic and biochemical studies conducted in schizophrenic patients. Indeed, mutations of the *Akt1* gene on chromosome 14q22–32 are associated with genetic form of schizophrenia (Harrison, 1999). Diminished Akt expression and kinase activity accompanied by reduced phosphorylation at Ser473 was found in postmortem brains of patients with schizophrenia (Kalkman, 2006; Zhao et al., 2006). Moreover, decreased expression of the catalytic subunit of PI3K was detected in lymphocytes of schizophrenic individuals (Middleton et al., 2005). These studies suggest that schizophrenia may be a consequence of decreased activity of the PI3K–Akt signaling cascade. Accordingly, some antipsychotic drugs result in the correction of dysfunctional PI3K–Akt signaling pathway (Chalecka-Franaszek and Chuang, 1999; Lu and Dwyer, 2005; Kalkman, 2006). Both PI3K and Akt are upstream regulators of mTORC1 (Kalkman, 2006), but whether mTORC1 is downregulated due to decreased activity of PI3K and/or Akt has not been clearly demonstrated in schizophrenia. Recently, the consequences of reduced Akt activity were investigated in a mouse model in which phosphorylation of Ser473 was abrogated selectively in neurons by genetic deletion of Rictor, the key regulatory subunit of mTORC2 (Siuta et al., 2010). Mice that lack mTORC2-dependent phosphorylation of Akt on Ser473 exhibited decreased pre-pulse inhibition, consistent with impairments in sensorimotor integration. These behavioral impairments were accompanied by alterations in cortical catecholamine content, as evidenced by decreased levels of dopamine (DA) and increased levels of norepinephrine (NE) in the PFC (Siuta et al., 2010). This finding is consistent with experimental models of schizophrenia that suggest that dysregulation in cortical DA content contribute both to cognitive deficits and negative symptoms (Weinberger et al., 1988; Davis et al., 1991; Howes and Kapur, 2009). The molecular link between decreased mTORC2–Akt signaling and abnormalities in DA levels in the PFC of Rictor null mice was explained by an Akt-dependent increase in surface expression and function of the NE transporter (NET), resulting in enhanced uptake of DA in noradrenergic neurons and its conversion to NE (Siuta et al., 2010). Finally, administration of nisoxetine, a NET inhibitor, reversed the sensorimotor deficits and the DA content in the PFC of the Rictor null mice, suggesting that the behavioral deficits displayed

by the mice are a consequence of an Akt-dependent imbalance in cortical levels of DA (Siuta et al., 2010).

Other evidence of the critical involvement of Akt signaling in schizophrenia comes from studies in which Akt activity is increased. For example, suppression of DISC1 activity results in increased phosphorylation of Akt and ribosomal protein S6, which is a downstream effector of mTORC1, during adult neurogenesis in the hippocampus (Kim et al., 2009). *DISC1* is a gene that was identified at the breakpoint of a chromosomal translocation (Blackwood et al., 2001; Chubb et al., 2008). Disruption of DISC1, due to genetic rearrangement, co-segregates with several psychiatric disorders, including schizophrenia (Millar et al., 2000; Blackwood et al., 2001). DISC1 regulates Akt by blocking KIAA1212, which is a binding partner of Akt (**Figure 1**). Therefore, deletion of DISC1 results in uncontrolled binding of KIAA1212 to Akt, leading to enhanced Akt signaling (Kim et al., 2009). Genetic manipulations that increase Akt activity resemble the defects caused by DISC1 suppression; importantly, these defects were rescued by inhibition of mTORC1 activity with rapamycin (Kim et al., 2009). These findings identified Akt–mTORC1 as a critical downstream target of DISC1 in neuronal development. It will be important to determine how these molecules, which have been indicated as schizophrenia-related genes, can contribute to the etiology of schizophrenia.

NEURODEGENERATIVE DISEASES

Parkinson's disease

Parkinson's disease is a progressive neurodegenerative disorder that results from loss of the dopaminergic (DAergic) neurons located in the substantia nigra pars compacta that project to the striatum (nigrostriatal DAergic pathway). The loss of striatal DA content leads to motor symptoms such as bradykinesia, rigidity, and tremor (Parkinson, 2002). Recent studies addressing the mechanism of neurodegeneration in PD demonstrate the involvement of the mTORC1 signaling pathway in the survival mechanism of DAergic neurons.

In vivo and *in vitro* studies show that degeneration induced by treatment with PD toxins, such as 6-OHDA and MPTP, leads to upregulation of RTP801, a protein encoded by a *RTP801* stress-responsive gene, which in turn reduces mTOR kinase activity. Importantly, enhanced RTP801 was also found in nigral DAergic neurons of postmortem human PD brains. Accordingly, neurons with inhibited expression of RTP801 or overexpression of mTOR were protected from the insults induced by the toxins and showed high survival rate (Malagelada et al., 2006). These studies suggest that neurodegeneration induced by PD toxins is dependent on upregulated RTP801 and the subsequent reduction of mTOR signaling.

It has been proposed that the molecular mechanism, linking high levels of RTP801 to mTORC1 inhibition and neurodegeneration involves TSC2 and Akt (Deyoung et al., 2008; Malagelada et al., 2008; **Figure 1**). Either genetic manipulations that interfere with TSC2 or increase the expression of a constitutively active form of Akt protected against the PD toxins and prevented the increase in RTP801 (Malagelada et al., 2008). These findings suggest that PD toxins induce neurodegeneration through inhibition of Akt, elevation of RTP801 levels, and TSC1/2-dependent suppression of mTORC1 activity. Importantly, DAergic neurons of PD patients also exhibited diminished levels of Akt phosphorylation together with increased expression of RTP801 (Malagelada et al., 2010). Thus, treatments that inhibit mTORC1 signaling should lead to an enhanced toxicity and even a greater DAergic loss. Surprisingly, rapamycin was reported as neuroprotective agent both in cell culture and in a MPTP mouse model. The beneficial effect of rapamycin, which inhibits mTORC1, has been explained through selective maintenance of mTORC1-dependent activities responsible for neuronal survival whereas RTP801, which acts upstream of mTORC1, would completely block all of its downstream physiological effects (Malagelada et al., 2010). For example, inhibition of mTORC1 may reduce the synthesis of new RTP801, thereby relieving RTP801-dependent inhibition of mTORC1 and stimulating the phosphorylation of the survival promoting kinase Akt (Malagelada et al., 2010). Alternatively, rapamycin may enhance Akt activity through inhibition of mTORC1-dependent activation of S6K1 and the subsequent reduction of phospho-IRS-1, which is a scaffold protein involved in the activation of PI3K and Akt (Shah et al., 2004).

The mTORC1 signaling cascade also was shown to be involved in the onset of levodopa-induced dyskinesia (LID), a debilitating side effect associated with the pharmacotherapy of PD (Santini et al., 2009b). LID consists of dystonic and choreic motor disturbances that are induced by repetitive and discontinuous administration of L-DOPA, the main therapy of choice for the treatment of PD (Bezard et al., 2001). A single administration of L-DOPA induces transient activation of mTORC1 signaling in a mouse model of PD (Santini et al., 2009b). Upregulated mTORC1 activity, measured by increased phosphorylation of downstream effectors, occurred in the dopamine D1 receptor (D1R) expressing subset of striatal neurons, specifically medium spiny neurons (MSNs), which were previously implicated as an important site for the onset and development of LID (Gerfen et al., 2002; Westin et al., 2007; Santini et al., 2008, 2009a; Darmopil et al., 2009). Accordingly, the ability of L-DOPA to activate mTORC1 signaling in D1R-bearing MSNs was maintained with repetitive administration of the drug and was correlated with the severity of the degree of dyskinesia. Treatment with rapamycin together with L-DOPA relieved the dyskinetic side effects without reducing the positive, anti-parkinsonian efficacy of drug (Santini et al., 2009b). These results suggest that inhibitors of mTORC1 may be used clinically as antidyskinetic agents. In summary, rapamycin and other inhibitors of mTORC1 have promising therapeutic potential for the treatment of PD, both as neuroprotective and antidyskinetic drugs.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion mutation of the trinucleotide CAG in exon 1 of the huntingtin (*HTT*) gene. The expansion mutation is translated into an abnormally long polyglutamine tract at the N terminus of huntingtin (MacDonald et al., 1993). The pathogenic mechanism by which huntingtin with an extended polyglutamine tract induces neuronal dysfunction and neurodegeneration is not fully understood. However, mutated huntingtin localizes in intraneuronal aggregates together with

other aggregate-prone proteins, and these inclusions appear to be deleterious for the neurons (Davies et al., 1997; Saudou et al., 1998; Rubinsztein, 2002). Turnover of huntingtin was found to be impaired after inhibition of the autophagy–lysosome pathway (Ravikumar et al., 2002) suggesting that this pathway is involved in the clearance of the intracellular inclusions. Activation of mTORC1 also inhibits autophagy (**Figure 1**) in cells from yeast to humans (Schmelzle and Hall, 2000). Consistent with this observation, induction of autophagy by inhibition of mTORC1 with rapamycin resulted in protection against mutated huntingtin-induced toxicity and enhanced clearance of aggregates. In the same cell line, the ability of rapamycin to inhibit mTORC1 and subsequently reduce inclusions was impaired after prolonged expression of mutated huntingtin (Ravikumar et al., 2002). The decreased effectiveness of rapamycin was explained with the localization of mTOR in the intracellular inclusions after prolonged expression of mutated huntingtin (Ravikumar et al., 2004). Co-localization of mTOR and mutated huntingtin to the inclusions was also described in the brain of transgenic mice expressing mutant huntingtin and, most importantly, in human patients affected by HD (Ravikumar et al., 2004). The interaction between mTOR and mutated huntingtin also was confirmed with co-immunoprecipitation in cultured cells and in brain lysates from mutant huntingtin transgenic mice (Ravikumar et al., 2004). Thus, mTOR and mutated huntingtin are sequestered together in intracellular inclusions, resulting in inactivation of mTORC1 as evidenced by reduced levels of phosphorylation of S6K1, 4E-BP2, and S6 both in cell culture and in the brains of the mutant huntingtin transgenic mice. In these conditions, mTORC1-dependent protein synthesis and autophagy were impaired and were not further reduced by treatment with rapamycin (Ravikumar et al., 2004). These findings suggest that inhibition of mTORC1 is effective in preventing toxicity induced by mutant huntingtin only in earlier stages of disease, possibly when mTOR is not yet sequestered and inactivated in the intracellular inclusions. Consistent with this idea, chronic administration of rapamycin to HD model mice before disease onset rescued the behavioral deficits associated with the progression of the disease (Ravikumar et al., 2004). HD model mice treated with rapamycin exhibited improvement in performance in four different behavioral paradigms used to test motor dysfunction: rotarod test, grip strength test, wire maneuver, and presence of tremors (Ravikumar et al., 2004). Moreover, the behavioral recovery in these mice was accompanied by a reduction in the number and size of striatal and cortical inclusions (Ravikumar et al., 2004). Taken together, these findings suggest that early pharmacological intervention to inhibit mTORC1 signaling effectively reduces neurological phenotypes in animal models of HD.

Alzheimer's disease

Alzheimer's disease (AD) is characterized by the formation of amyloid plaques and neurofibrillary tangles (NFTs), which are correlated with neurodegeneration in brain areas implicated in acquisition and storage of declarative memory, such as the hippocampus, entorhinal cortex, amygdala, and associative regions of the cortex (Nestor et al., 2006; Götz and Ittner, 2008). The major component of plaques is the polypeptide Aβ that is derived from the amyloid precursor protein (APP), whereas NFTs are composed of hyperphosphorylated forms of the microtubule-associated protein tau (Glenner and Wong, 1984; Kang et al., 1987; Tanzi et al., 1987; Binder et al., 2005).

Studies on the regulation of the mTORC1 signaling pathway in AD has revealed a complicated picture, mainly due to the lack of agreement about the direction of mTORC1 dysfunction (increase vs. decrease) and the pathogenic mechanism leading to neurodegeneration (Swiech et al., 2008). Aβ and tau exert toxicity through distinct mechanisms but there is now evidence from human, rodent, and *in vitro* studies demonstrating a link between Aβ and tau in neurotoxicity, although the molecular nature of this link remains elusive (Ittner and Götz, 2011). Studies of Aβ-induced neurotoxicity report increased phosphorylation of double-stranded RNA-dependent protein kinase (PKR; Chang et al., 2002a,b; Peel and Bredesen, 2003; Onuki et al., 2004), eukaryotic initiation factor 2α (eIF2α; Chang et al., 2002a,b) and S6K1 (An et al., 2003) in mouse models of AD and AD patients. For example, the Aβ peptide is shown to activate PKR and Aβ-induced neurotoxicity was reduced in neuronal cultures from PKR knockout mice. (Chang et al., 2002b) Furthermore, increased levels of phosphorylated PKR were found in APP/PS1 mutant mice, a rodent model of AD expressing human APP with the Swedish and London mutation, as well as presenilin 1 (PS1) carrying two different mutations known to cause a familial, early onset form of the disease (Page et al., 2006). These studies suggest a correlation between Aβ-induced neurotoxicity and a decrease in translation, as PKR attenuates protein synthesis through eIF2α phosphorylation. In support of the idea that AD is accompanied by reduction of protein translation, the mTORC1 signaling cascade was found to be impaired in a mouse model of AD and was correlated with impaired synaptic plasticity (Ma et al., 2010). It was possible to mimic these impairments by treating hippocampal slices of wild-type mice with soluble Aβ. Importantly, pharmacological and genetic manipulation leading to increased mTORC1 signaling restored the synaptic plasticity deficits of the AD model mice and prevented Aβ-induced impairments in synaptic plasticity in wild-type mice (Ma et al., 2010). Altogether, these data indicate that there is an association between Aβ-induced toxicity, enhanced eIF2α phosphorylation, and deficits in mTORC1 signaling pathway.

On the other hand, several groups have reported increases in the phosphorylation of S6K1, mTOR, eIF4E, and 4E-BP2 in AD brains (Li et al., 2004, 2005). This led to the hypothesis that enhanced mTORC1 signaling could be responsible for the high levels of toxic proteins such as tau (Pei et al., 2008). In addition, hyperactivated mTORC1 signaling was also positively correlated with the levels of total and phosphorylated tau. Thus, the continuous synthesis of tau in degenerating neurons could be the result of upregulated mTORC1 signaling (Pei and Hugon, 2008). Furthermore, increased levels of phosphorylated mTOR and S6K1 were also found in some of the brain areas affected in AD, such as cortex, in the same double APP/PS1 transgenic mice (Lafay-Chebassier et al., 2005). Importantly, chronic treatment with rapamycin rescued learning and memory deficits in two different mouse models of AD (Caccamo et al., 2010; Spilman et al., 2010) suggesting that enhanced mTORC1 activity is involved in the cognitive impairments.

PSYCHOACTIVE DRUGS

The use of the term psychoactive when describing drugs refers to a diverse group of chemicals that act primarily at the level of the central nervous system and affect brain function, resulting in changes of mood, cognition, perception, and consciousness. These substances are often used recreationally even if originally synthesized for the therapeutic use, namely to treat both physical and neuropsychiatric disorders (Di Chiara and North, 1992; Nestler, 2004). Recently, studies have demonstrated that discrete behavioral effects induced by some of these drugs are caused by activation of the mTORC1 signaling pathway in the brain.

Cannabinoids

Cannabis sativa is included in marijuana, hashish, bhang, and other well-known illicit psychoactive drugs that are largely used recreationally (Pierce and Kumaresan, 2006). The primary psychoactive ingredient of cannabis is Δ^9-tetrahydrocannabinol (THC; Gaoni and Mechoulam, 1964), which is responsible for multiple behavioral effects including euphoria, enhanced sensory perception, increased appetite, analgesia, memory impairment, anxiety, paranoia and, at higher doses, sedation (Ameri, 1999; Piomelli, 2003; Pierce and Kumaresan, 2006). Experimental mouse models are utilized to study behaviors induced by THC (Hampson and Deadwyler, 1999; Maldonado and Rodriguez De Fonseca, 2002). For example, acute administration of THC to mice immediately after training in the novel object recognition task produced impairment in long-term memory, as indicated by the inability of THC-treated mice to discriminate between familiar and novel objects (Puighermanal et al., 2009). Moreover, the same dose of THC activated the mTORC1 signaling cascade in the hippocampus, as indicated by increased phosphorylation of downstream targets, such as S6K1, S6, eIF4E, eIF4G, and 4E-BP2 (Puighermanal et al., 2009). The behavioral and molecular changes induced by THC were abolished in CB1 receptor knockout mice and in mice with a conditional deletion of the CB1 receptor in GABAergic neurons. Moreover, pretreatment of mice with either rapamycin or anisomycin abolished the amnesic-like effects induced by THC, suggesting that the memory impairment is mediated by mTORC1-dependent translation (Puighermanal et al., 2009). Surprisingly, the THC-induced activation of the mTORC1 cascade was localized in glutamatergic pyramidal neurons and was dependent on NMDA receptors (Puighermanal et al., 2009). This indicates that the amnesiac-like effect induced by THC is most likely mediated via a heterosynaptic mechanism because CB1 and NMDA receptors are localized in different hippocampal neurons (GABAergic basket-cells and glutamatergic pyramidal neurons, respectively). It is also interesting to note that the same molecular process of protein translation occurring in hippocampal pyramidal neurons, is involved in opposing behavioral processes; memory consolidation vs. THC-induced memory loss. A possible explanation for the contradictory functional effects of translation may be due to the different synaptic locations where it occurs (Mackie and Katona, 2009). Indeed, in pyramidal neurons THC-induced activation of mTORC1, and potentially translation, occurs in the stratum pyramidale, a cellular region with a high density of GABAergic synapses (Papp et al., 2001). Thus, it is possible that enhanced translation in the soma of pyramidal neurons results in memory impairments, whereas enhanced translation in dendritic spines at excitatory synapses is required for memory formation (Costa-Mattioli et al., 2009b; Richter and Klann, 2009).

Cocaine

Cocaine recently was shown to activate the mTORC1 signaling cascade in brain regions that undergo long-lasting neuroadaptation in response to drug exposure, including the mesolimbic dopamine pathway (VTA) and its forebrain targets, especially the nucleus accumbens (NAc; Nestler, 2005; Mameli et al., 2007; Wang et al., 2010; Wu et al., 2011). Specifically, systemic administration of cocaine in mice increased the phosphorylation of the mTORC1 downstream effector S6 in the VTA and NAc (Wu et al., 2011). Sensitization to cocaine is often measured in behavioral experiments as an increase in locomotor activity. Behavioral sensitization consists of an initial induction phase, in which repeated injections of cocaine leads to an increase in locomotor activity. Induction is followed by drug withdrawal and then re-exposure to the drug for the expression phase of sensitization, which results in an even greater enhancement in locomotor activation (Berridge and Robinson, 1998). Rapamycin effectively reduced both induction and expression of cocaine-induced locomotor sensitization (Wu et al., 2011), suggesting that mTORC1 signaling may underlie the different adaptive changes elicited by cocaine exposure during induction and expression phases of locomotor sensitization. Notably, it also has been shown that cocaine induces altered synaptic plasticity in DA neurons of the VTA in an mTORC1- and protein synthesis-dependent manner (Mameli et al., 2007). These findings provide additional evidence for cocaine-induced activation of mTORC1.

Another aspect of cocaine addiction linked to activation of mTORC1 signaling is relapse, which is experienced by addicts after a prolonged period of abstinence and induced by craving-provoking environmental cues paired with the rewarding properties of the drug (Gawin and Kleber, 1986a,b; O'Brien, 1997; Childress et al., 1999; Wang et al., 2010). Cue-induced relapse is modeled in rodents with a reinstatement paradigm, in which rats are trained to self-administer a drug reward paired with a discrete cue. Lever pressing is then extinguished in the absence of the discrete cue. During a reinstatement test, exposure to the discrete cue reinstates drug-seeking behavior as measured by increased lever pressing (Meil and See, 1997; Kruzich and See, 2001). Reinstatement of drug-seeking behavior in rats exposed to a cue previously associated with the drug was accompanied by an increase of mTORC1 activation in the NAc, as measured by increased phosphorylation of S6K1 and S6 (Wang et al., 2010). Infusion of rapamycin in the NAc core before the reinstatement test significantly decreased the cue-induced seeking behavior (Wang et al., 2010). These behavioral effects are NMDA-dependent as NMDA agonist infusions into the NAc core enhanced the reinstatement of cue-induced cocaine seeking by increasing the activation of the mTORC1 cascade, and co-infusions of rapamycin blocked both the molecular and behavioral effects (Wang et al., 2010). These findings suggest that mTORC1-dependent protein synthesis in the NAc core is involved in reinstatement of cue-induced seeking behavior. Because rapamycin administered acutely before the reinstatement test reduces the cue-induced cocaine seeking, it could used therapeutically to treat relapse in cocaine addicts (Wang et al., 2010).

Importantly, it has been shown that rapamycin reduced the craving induced by a drug-related cue in abstinent heroin addicts (Shi et al., 2009).

CONCLUSION AND FUTURE DIRECTIONS

It is clear that mTORC1 is a critical regulator of multiple functions of the central nervous system. In this review, we have described a series of disorders in which there is a clear association between neuropathology and regulation of mTORC1-dependent translation (see **Table 1** for a summary).

However, mTORC1 has several other physiological functions that either are not fully understood or have not yet been explored. For instance, the role of dysregulated mTORC1 signaling in autophagy in neurons, and its association with diseases has the potential to open a completely new line of research. Furthermore, there are aspects of cap-dependent protein synthesis that are regulated via mTORC1 that deserve more attention and/or need to be addressed in more detail. For example, the molecular machinery that is involved in the sorting and localization of particular mRNAs at specific neuronal subregions is poorly understood, as is the identity of the mRNAs whose translation is regulated by mTORC1.

An open question that needs to be addressed in greater detail is the role that mTORC1 plays in the pathophysiology of the CNS.

Table 1 | Summary of altered mTORC1 signaling in brain disorders and in response to psychoactive drugs.

Disorder	mTORC1 signaling	Readout	Brain regions examined	Impact of rapamycin on behavior	Reference
Fragile X syndrome	↑	p-mTOR, p-4E-BP, p-S6K1, mTOR/Raptor, eIF4E/eIF4G interactions	Hippocampus, cortex	Not examined	Sharma et al. (2010)
Tuberous sclerosis complex	↑	p-S6	Hippocampus	Rescue of deficits in spatial memory (Morris water maze) and context discrimination	Ehninger et al. (2008a)
PTEN mutation (autism)	↑	p-S6	Hippocampus	Rescue of impaired social behaviors, seizures, and macrocephaly	Kwon et al. (2006a), Zhou et al. (2009)
Depression	↓	Ketamine-induced p-mTOR, p-S6K1, and p-4E-BP	Prefrontal cortex	Blockade of antidepressant effects of ketamine (forced swim and novelty suppressed feeding tests)	Nanxin et al. (2010)
Schizophrenia	↑	p-S6	Hippocampus	Not examined	Kim et al. (2009)
Parkinson's disease	↓ in PD ↑ in l-DOPA-induced dyskinesia	p-S6K1, p-S6, p-4E-BP, and p-eIF4E	Striatum	Reduction of l-DOPA-induced dyskinesia	Malagelada et al. (2006), Santini et al. (2009b)
Huntington's disease	↓	p-S6K, p-S6, and p-4E-BP	Striatum and cortex	Improvement of performance in rotarod, grip, and wire tests; reduction of tremor	Ravikumar et al. (2004)
Alzheimer's disease	↑ ↓	p-mTOR, p-S6K1, p-4E-BP, and p-eIF4E	Cortex, hippocampus, cerebellum	Rescue of spatial memory (Morris water maze)	An et al. (2003), Li et al. (2004), Lafay-Chebassier et al. (2005), Caccamo et al. (2010), Spilman et al. (2010)
Cannabis (THC)	↑	p-S6K1, p-S6, p-eIF4E, p-eIF4G, p-4E-BP	Hippocampus	Blockade of amnesic-like effects of THC (novel object recognition and context discrimination tests)	Puighermanal et al. (2009)
Cocaine	↑	p-S6K, p-S6	Ventral tegmental area (VTA), nucleus accumbens	Reduction of cocaine-induced locomotor sensitization and cue-induced seeking behavior	Wu et al. (2011), Wang et al. (2010)

The ubiquitous distribution and the fine control of mTORC1 signaling render it difficult to ascertain whether mTORC1 is the cause and/or one of the consequences of various brain disorders discussed in this review. At minimum, we would argue in favor of a causative role of mTORC1 in the etiology of brain disorders where behavioral abnormalities are corrected either by pharmacological or genetic manipulations of mTORC1.

Most research investigating mTOR signaling in the brain is focused on mTORC1, with very little known about mTORC2. The contribution of mTORC2 to the functions of the central nervous system needs to be clarified. Moreover, since mTOR is a component of both mTORC1 and mTORC2, there may be a dynamic equilibrium regarding the relative amount of mTOR engaged in the two mTORCs. Furthermore, it will be important to understand whether there is interplay between mTORC1 and mTORC2 during normal physiological processes in the brain, in neurological disorders and/or in response to psychoactive drugs.

Finally, the mTORC1 signaling pathway and its impact on protein synthesis is well characterized in some brain regions and cell types such as glutamatergic pyramidal neurons of the hippocampus, but it has not been rigorously explored in other regions. For example, mechanisms of translational control in striatal GABArgic neurons and/or glial cells have not been studied intensely. Thus, in the future, more thorough studies of the mechanisms that regulate translation will be necessary in diverse brain regions and cell types in order to acquire a better understanding of how proteins are synthesized throughout the brain in response to diverse stimuli.

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Modulation of neuronal signal transduction and memory formation by synaptic zinc

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The physiological role of synaptic zinc has remained largely enigmatic since its initial detection in hippocampal mossy fibers over 50 years ago. The past few years have witnessed a number of studies highlighting the ability of zinc ions to regulate ion channels and intracellular signaling pathways implicated in neuroplasticity, and others that shed some light on the elusive role of synaptic zinc in learning and memory. Recent behavioral studies using knock-out mice for the synapse-specific zinc transporter ZnT-3 indicate that vesicular zinc is required for the formation of memories dependent on the hippocampus and the amygdala, two brain centers that are prominently innervated by zinc-rich fibers. A common theme emerging from this research is the activity-dependent regulation of the Erk1/2 mitogen-activated-protein kinase pathway by synaptic zinc through diverse mechanisms in neurons. Here we discuss current knowledge on how synaptic zinc may play a role in cognition through its impact on neuronal signaling.

Keywords: synaptic zinc, memory, neuronal plasticity, signal transduction

INITIAL IDENTIFICATION OF VESICULAR ZINC IN SYNAPTIC TERMINALS OF THE BRAIN

The first indication of the accumulation of zinc in nerve fibers came from Maske (1955). He found that intravital injection of the heavy metal chelator dithizone results in a band of bright red staining – corresponding to metal: dithizonate – in the mossy fiber region of the hippocampus. Subsequently, histochemical methods were developed (i.e., Timm's stain) to precipitate metal cations *in situ* and visualize them by silver amplification (Timm, 1958; Danscher, 1981). When combined with electron microscopy, these methods revealed the precise localization of metal ion staining within synaptic vesicles of the telencephalon (**Figure 1A**; Haug, 1967; Perez-Clausell and Danscher, 1985). Suggesting the involvement of zinc in the vesicular staining, autoradiographic and analytical studies had shown high levels of zinc in the mossy fiber region (Dencker and Tjalve, 1979; Frederickson et al., 1983). Indeed, proton-induced X-ray emission spectroscopy identified zinc as the main ion detected by the above histochemical techniques in brain samples (Danscher et al., 1985). More recently, membrane-permeable fluorophores with good selectivity for zinc ions (such as TSQ, ZP-1, ZnAF-2, or ZnIC, to name a few) have confirmed the abundance of zinc in synaptic terminal fields (**Figure 1B**; Frederickson et al., 1987; Ueno et al., 2002; Woodroofe et al., 2004; Komatsu et al., 2007).

Pre-synaptic zinc is observed in all the vertebrates that have been examined (Frederickson, 1989). Also common between species is the remarkable neuroanatomical distribution of pre-synaptic zinc, which is greatly restricted to cortical and limbic regions of the brain in a distinct laminar pattern (**Figure 1C**). Vesicular zinc is also present in boutons of the olfactory bulb and spinal cord (Friedman and Price, 1984; Birinyi et al., 2001; Danscher et al., 2001). In contrast with the distribution of vesicular zinc, absolute zinc levels are relatively even across the gray matter (60–80 ppm; Frederickson et al., 1983). The apparent disparity between absolute and pre-synaptic zinc levels stems from the fact that about 80% of total zinc in brain is not amenable to histochemical detection (Cole et al., 1999). This "invisible" pool of zinc is found in all six classes of enzymes and in intracellular receptors, where it is normally coordinated by four to six ligands, frequently supplied by histidine and cysteine, in conserved binding motifs (Vallee and Falchuk, 1993; Auld, 2001).

Zinc uptake into synaptic vesicles requires the zinc transporter protein ZnT-3, which is exclusively sorted to clear synaptic vesicles (Palmiter et al., 1996; Cole et al., 1999). Accordingly, zinc staining is excluded from dense core vesicles in pre-synaptic boutons (Perez-Clausell and Danscher, 1985). Zinc staining of synaptic vesicles is evenly distributed at various distances from the active zone (Lavoie et al., 2007), suggesting that both ready-releasable and reserve vesicle pools may contain vesicular zinc. In keeping with this, all synaptic vesicles appear to stain for ZnT-3 in labeled boutons (Wenzel et al., 1997). The fact that only a fraction of these vesicles stains for zinc may reflect a sensitivity issue or a requirement for additional proteins to uptake zinc into vesicles (see next section). In the telencephalon, all boutons stocked with vesicular zinc (i.e., zincergic) establish asymmetric synapses that typically involve dendritic spines (Perez-Clausell and Danscher, 1985). As predicted from their ultrastructure, these zincergic boutons are glutamatergic (Martinez-Guijarro et al., 1991; Beaulieu et al., 1992; Sindreu et al., 2003) and are apposed to post-synaptic membranes enriched in glutamate receptor subunits (Sindreu et al., 2003). In contrast, zincergic boutons in the spinal cord often form symmetric synapses immunoreactive to γ-aminobutyric acid (Danscher et al., 2001). The zincergic MF terminals of the hippocampus represent a special case, as they are both glutamatergic and GABAergic (Ruiz et al., 2004). A common observation in electron microscopy studies is that only a fraction of excitatory synapses show vesicular

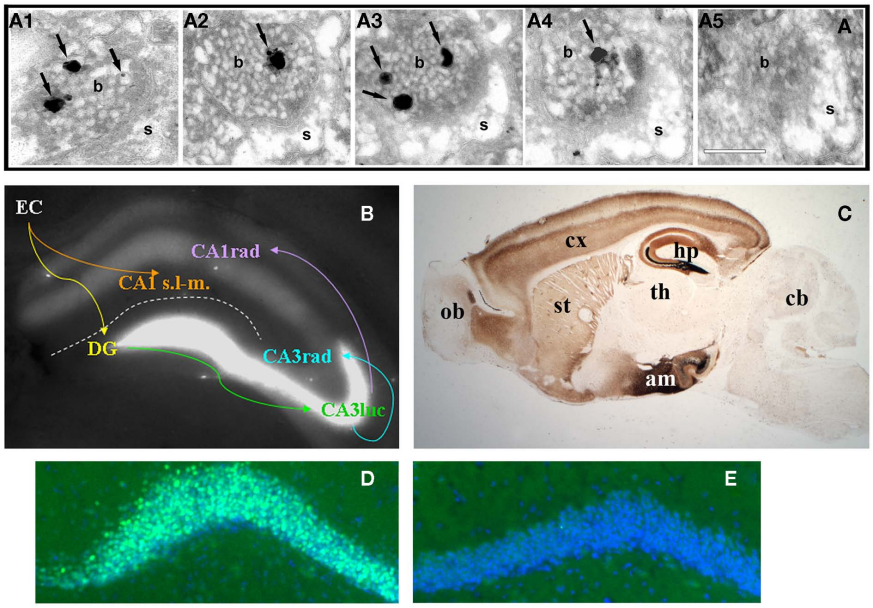

FIGURE 1 | (A) Consecutive serial sections (A1–5) of an axospinous synapse in CA1 stratum radiatum stained for vesicular zinc using the Neo-Timm method and observed by electron microscopy. Arrows point to silver granules of varying size detecting reactive zinc in vesicles. Note the presence of zinc staining in four of five profiles. b, bouton; s, spine. Scale bar, 200 nm. **(B)** Zinpyr-1 fluorescence in a fresh hippocampal slice with major hippocampal connections indicated by arrows. Reactive zinc is highest in stratum lucidum (luc) of CA3, moderate in stratum radiatum (rad), and absent in stratum lacunosum-moleculare (s.l-m.) of CA1. **(C)** Bright field image of a sagittal brain section from a 5 month-old C57/BL6J mouse stained with the Neo-Timm method. ob, olfactory bulb; cx, cortex; st, striatum; th, thalamus; hp, hippocampus; am; amygdala; cb, cerebellum. **(D,E)** Beta-galactosidase immunofluorescence (green) in dentate gyrus granule cells of a ZnT-3 KO **(D)** and control **(E)** mouse, counterstained with Hoescht (blue). C. Sindreu, unpublished observations.

zinc staining. Studies quantifying the incidence of vesicular zinc or ZnT-3 among axospinous synapses are scarce, but measurements in the CA1 region suggest that about half of Schaffer-collateral synapses may be zincergic (Sindreu et al., 2003). This raises the question as to whether zincergic boutons arise from a subset of glutamatergic neurons or instead only a subset of boutons from any given neuron may be zincergic. Retrograde tracing studies strongly support the former possibility, as parent neurons of zincergic boutons constitute a subpopulation of neurons in the brain, and they are often interspersed with non-zincergic neurons of similar morphology (Slomianka et al., 1990, 1997; Slomianka, 1992; Brown and Dyck, 2004; Cunningham et al., 2007). The hippocampal MFs are a notable exception to this rule: all giant terminals are zincergic and hence all mature dentate gyrus granule cells may give rise to zincergic terminals. This can be appreciated by the expression of a β-galactosidase reporter in virtually all granule cells when driven by the ZnT-3 promoter (**Figures 1D,E**). Double-tracing studies have further confirmed that the vast majority of zincergic boutons emanate from pyramidal cells in the telencephalon (Miro-Bernie et al., 2003). Interestingly, neuronal projections from or to subcortical structures are largely devoid of pre-synaptic zinc. Instead, zincergic projections selectively (and often reciprocally) inter-connect cortical and limbic structures (Mandava et al., 1993; Long et al., 1995; Christensen and Frederickson, 1998; Casanovas-Aguilar et al., 2002). This suggests that the zincergic connectome represents a subnetwork of intrinsic projections embedded in the general cortical system.

From a cognitive perspective, the abundance of vesicular zinc in synapses of the cortex, hippocampus, and amygdala suggests a possible role in learning and memory. Indeed, these three structures have been strongly implicated in memory formation, storage, and retrieval (Martin et al., 2000). Because activity-dependent changes in synaptic strength are widely assumed to support memory (Neves et al., 2008), several studies have also examined the effects of zinc on synaptic ion channels, receptors, and plasticity. Comparatively less is known as to the role that zinc plays on neuronal signal transduction, which controls the expression of long-term synaptic plasticity and underlies memory consolidation. At any level, a full understanding of the role that synaptic zinc plays in learning and memory requires identification of the different zinc reservoirs and mechanisms of zinc homeostasis operating at synapses.

HOMEOSTASIS OF SYNAPTIC ZINC

Several lines of evidence indicate the existence of multiple transmembrane zinc gradients among the main constituents the synapse (**Figure 2**).

PLASMA MEMBRANE

Using a carbonic anhydrase-based biosensor, the concentration of loosely bound or free zinc (i.e., reactive zinc) was estimated to be 5–20 nM in the extracellular brain fluid (Frederickson et al., 2006b). Given that total zinc in the cerebrospinal fluid is about 150 nM (Hershey et al., 1983), this value suggests that most extracellular

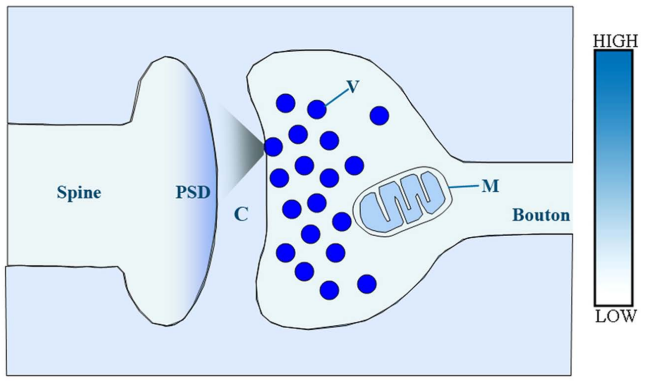

FIGURE 2 | Schematic illustration of presumed relative concentrations of reactive zinc in a central glutamatergic synapse. In this model, relative zinc levels are orientatively coded by color intensity. V, synaptic vesicle; M, mitochondrion; C, synaptic cleft; PSD, post-synaptic density.

zinc also is tightly bound. Establishing the concentration of cytosolic reactive zinc in neurons has been hampered by the technical limitations of using fluorescent indicators for intracellular zinc measurements, as discussed elsewhere (Nolan and Lippard, 2009; Vinkenborg et al., 2010). Attempts in cortical neuron cultures using the dye mag-fura-5 suggested that cytosolic zinc may be found at subnanomolar levels (Sensi et al., 1997). Similar estimates have been obtained in non-neuronal cells with different indicators: ∼0.6 nM Zn^{2+} in HT293 cells by using FluoZin-3 (Krezel and Maret, 2006), and ∼0.4 nM Zn^{2+} in INS-1(832/13) β-cells by using eCALWYs FRET-based sensors (Vinkenborg et al., 2009). Together, the data suggest that the concentration of reactive zinc may be an order of magnitude lower in the cytosol compared with the extracellular brain space. This is noteworthy in view of the fact that the total intracellular zinc concentration largely exceeds that in the extracellular space (∼150 μM; Ehmann et al., 1984). Thus, reactive zinc in the cytosol may represent less than 0.001% of total cellular zinc. Attesting to the importance of controlling the levels of cytosolic zinc, prolonged increases or reductions in intra- or extracellular zinc can compromise neuronal viability (Canzoniero et al., 1999; Bozym et al., 2010).

How circulating zinc, previously absorbed in the gastrointestinal tract, enters the brain or CSF is poorly understood, although it appears to involve active transport mechanisms (Takeda, 2000). Likewise, the homeostatic mechanisms maintaining the zinc gradient across the plasma membrane in neurons are poorly defined, and currently none of the candidate proteins have been directly localized to synapses. The principal mechanism for zinc extrusion in neurons appears to be a secondary active transporter dependent on the Na^{+} and Ca^{2+} electrochemical gradients (Ohana et al., 2004; Qin et al., 2008), but the identity of this exchanger remains elusive. Given that neuronal activity elicits voltage-gated ion fluxes across the plasma membrane, such a zinc extrusion mechanism could potentially be subject to activity-dependent regulation. The zinc transporter ZnT-1 of the Slc30a family was the first plasma membrane protein found to regulate zinc extrusion (Palmiter and Findley, 1995). ZnT-1 is expressed in neurons and glia in several regions of the brain, including some that lack vesicular zinc (Sekler et al., 2002), and its genetic deletion is embryonic lethal (Andrews et al., 2004). Knock-down of ZnT-1 in cultured neurons decreases the rate of zinc efflux (Qin et al., 2009), but it is unclear if ZnT-1 affects the resting level of cytosolic zinc in the presence of an inwardly directed zinc gradient. ZnT-1-dependent zinc efflux is not affected by extracellular cations or ATP depletion, and it may involve oligomerization or association with other proteins (Palmiter and Findley, 1995). In addition, ZnT-1 suppresses the influx of other divalent cations via voltage-gated calcium channels (Ohana et al., 2006; Levy et al., 2009), suggesting additional roles besides zinc transport.

On the other hand, the ZIP-1 and -3 members of the Slc39a protein family have been implicated in the tonic import of extracellular zinc into neurons. ZIP-1 and -3 are highly expressed in CA1 hippocampus, and pyramidal neurons from double-knockout mice show ∼50% reduction in the amount of extracellular zinc uptake (Qian et al., 2011). Assuming that zinc extrusion mechanisms are intact in this mutant mouse, the data indicate a major contribution of ZIP-1 and -3 to neuronal zinc import. The mechanism of zinc transport by ZIP proteins is not fully understood, although it was found to be energy-independent (Gaither and Eide, 2000). Evidence from the bacterial homolog ZIPB indicates that ZIP proteins may act as non-saturable zinc channels allowing passive zinc entry down its concentration gradient (Lin et al., 2010). ZIPB-dependent zinc entry increases with the proton concentration, in keeping with the effect of intracellular pH on neuronal zinc import (Colvin, 2002). Intriguingly, earlier studies found that uptake of Zn^{65} into the neuropil was energy-dependent (Howell et al., 1984), implying that alternative or additional mechanisms may import zinc at synapses.

There is also an interest in the role that the small Zn/Cu-binding protein metallothionein-3 (i.e., MT-3) may play on cytosolic zinc homeostasis. MT-3 has many remarkable biochemical properties with regards to the binding and release of zinc (Maret, 2011), and MT-3 over-expression confers resistance to zinc toxicity *in vitro* (Palmiter, 1995). An elegant study showed that MT-3 may confer neuroprotection from excitotoxicity by releasing zinc following phosphorylation by PKC, leading to downstream gene expression (Aras et al., 2009). However, MT-3 KO mice thrive under normal conditions and seem to respond normally to systemic zinc overload (Erickson et al., 1997). Hence, the physiological role of neuronal MT-3 in zinc homeostasis is unclear. A safety mechanism that may help maintain the zinc gradient across the plasma membrane is the sequestration of excess zinc into lysosomes. Thus, in CA3 neurons, kainate-induced seizures trigger the accumulation of zinc into lysosomes (Hwang et al., 2008) as well as the up-regulation of the zinc importer ZIP-4 (Emmetsberger et al., 2010). Suggesting a possible link between the two, endocytosis inhibitors prevent the lysosomal degradation of surface ZIP-4 that occurs during zinc overload (Mao et al., 2007).

POST-SYNAPTIC DENSITY

Biochemically isolated post-synaptic densities (PSD) contain a high amount of zinc (∼4 nmol/mg protein) that supports the structural organization of the PSD (Jan et al., 2002; Baron et al., 2006). Recent data indicate that at least a fraction of this zinc might

be reactive or exchangeable, as it can be detected in association with Shank2/3 scaffold protein aggregates in dendritic spines with fluorescent zinc indicators (Grabrucker et al., 2011). In line with this, the same study also showed that zinc chelators can quickly alter the ultrastructural appearance of the PSD in cultured neurons.

SYNAPTIC VESICLES

Direct measurements of the concentration of reactive zinc in synaptic vesicles are lacking, but simple calculations indicate that it could easily reach high micromolar levels. A single zinc atom would result in a concentration of $\sim$40 μM within a 0.64×10^{-5} μm^3 synaptic vesicle, which is the average vesicle volume in CA1 boutons (Schikorski and Stevens, 1997). The Neo-Timm's staining method has been claimed to be able to detect as little as 3–4 zinc atoms (Danscher and Stoltenberg, 2006), which would translate into a minimal concentration of $\geq$150 μM vesicular zinc. Along these lines, a concentration of $\sim$1 mM vesicular zinc was inferred by extrapolating from the amount of total zinc found in the hilar region of the hippocampus (Frederickson et al., 1983). On balance, it appears that reactive zinc is several-fold more concentrated within synaptic vesicles than in the cytosol or the extracellular space. The different concentrations likely account for the preferential labeling of synaptic vesicles with zinc probes. Intra-vesicular pH may also affect zinc speciation, releasing it from protein complexes therein and making it more reactive to zinc probes (Vinkenborg et al., 2009). By comparison, the concentration of glutamate in synaptic vesicles ranges from 60 to 210 mM (Clements, 1996). Interestingly, studies in PC12 cells indicate a functional coupling between the vesicular uptake of zinc and glutamate; recombinant murine VGLUT-1 and ZnT-3 were targeted to the same population of synaptic-like microvesicles and reciprocally facilitated their transport activities, perhaps through effects on the transvesicular electrochemical gradient (Salazar et al., 2005). In keeping with current estimates of the cytosolic zinc concentration, ZnT-3 enhanced VGLUT-1-mediated glutamate uptake at low nanomolar zinc levels. Whether ZnT-3 and VGLUT-1 cooperate in a similar manner in synaptic vesicles of the brain has not been examined. However, destroying the vesicular proton gradient with the H^+-ATPase inhibitor concanamycin A, which prevents glutamate uptake, also reduces pre-synaptic fluorescence of the zinc indicator Zinpyr-1 at putative single MFs (Ketterman and Li, 2008). Intriguingly, functional differences between human and mouse ZnT-3 have been reported. Thus, human, but not mouse, ZnT-3 tends to form dimmers that promote vesicular targeting and stimulate zinc uptake (Salazar et al., 2009).

The concentration of zinc in synaptic vesicles has led to the natural hypothesis that zinc may be co-released with glutamate into the synaptic cleft following vesicular exocytosis. Evidence that neuronal depolarization increases the extracellular level of elemental zinc, either *in vivo* or *in vitro*, and in parallel with glutamate, is overwhelming (Howell et al., 1984; Charton et al., 1985; Aniksztejn et al., 1987; Minami et al., 2002). However, no study has yet confirmed the synaptic vesicle origin of this zinc by performing similar experiments in ZnT-3 knock-out mice (which lack any reactive zinc within synaptic vesicles; Cole et al., 1999; Linkous et al., 2008). Other studies have addressed the issue of vesicular zinc release by stimulating brain slices in the presence of fluorescent zinc indicators. Thus, action potential-evoked zinc exocytosis has been reported in CA3 and CA1 synapses by detecting changes in FluoZin-3 fluorescence, an extracellular zinc dye (Qian and Noebels, 2005, 2006; Carter et al., 2011). Importantly, zinc release was found to be highly sensitive to changes in release probability, dependent on vesicular exocytosis, and it was ablated in ZnT-3 KO mice. These are remarkable observations considering the potential limitations of the approach (e.g., *ex vivo* zinc depletion, inadequate metal contamination or temperature, small fractional volume of the synaptic cleft, optical sensitivity, perturbation of zinc kinetics by the indicator, etc.). Indeed, mixed results have been obtained when using maximal stimulation to compensate for low optical resolution (Li et al., 2001b; Kay, 2003; Datki et al., 2007). The extracellular zinc increase associated with exocytosis should be paralleled by a corresponding decrease in vesicular zinc, and there are some zinc fluorescence measurements from MFs suggesting that this is also the case (Ketterman and Li, 2008). The results suggest a model where released zinc may diffuse into the synaptic cleft, transiently increasing its extracellular concentration in an activity-dependent manner. In keeping with this, vesicular zinc release has been shown to modulate post-synaptic receptors at the MF synapse (Vogt et al., 2000; Pan et al., 2011, see section below). Even more strikingly, intracellular zinc increases in post-synaptic neurons were recently reported following intense axonal depolarization (Suh, 2009; Carter et al., 2011). Again, ZnT-3 deletion abolished the post-synaptic zinc transient, suggesting that vesicular zinc can be trans-synaptically mobilized under some conditions. One intriguing observation from these experiments is the slow decay of the extracellular zinc transient that is triggered by exocytosis. Whether this is a property of individual synapses is still unknown, as zinc transients were not induced with minimal stimulation protocols to activate single MF boutons. The factors governing the synaptic diffusion and clearance of released zinc are not understood. It has been proposed that vesicular zinc may remain bound to synaptic proteins after exocytosis, although the putative synaptic transporters or proteins that may act as fixed buffers are undefined (Vogt et al., 2000; Kay, 2003). It has also been proposed that the high tortuosity of the MF synapse may delay the clearance of zinc (Qian and Noebels, 2005). Direct comparisons of the fluorescence kinetics shown by extracellular indicators for zinc and glutamate (Hires et al., 2008) after release have not been reported.

MITOCHONDRIA

Another potential source of synaptic zinc is that originating from mitochondria. Virtually all MF terminals and about half of small pre-synaptic boutons contain at least one mitochondrion (Ventura and Harris, 1999; Chang et al., 2006), where it can modulate synaptic plasticity by handling Ca^{2+} and supplying ATP (Cai et al., 2011). Recent evidence in neurons indicates that resting mitochondria may harbor two to threefold higher concentrations of reactive zinc relative to the cytosol (Dittmer et al., 2009). Zinc appears to access the mitochondrial matrix mainly via the Ca^{2+} uniporter, a process dependent on the mitochondrial membrane potential (Malaiyandi et al., 2005; Caporale et al., 2009). The mechanism whereby mitochondrial zinc is extruded is unknown, as no zinc

transporters have been localized to mitochondria. *In vitro* data suggest that large intracellular Ca^{2+} increases, often associated with neuronal injury, may possibly couple to mitochondrial zinc release into the cytosol (Sensi et al., 2003; Dineley et al., 2008; Dittmer et al., 2009).

SYNAPTIC ZINC AND MEMORY

The subsynaptic distribution of reactive zinc suggests three possible modes whereby synaptic zinc could affect cognitive processes: (i) tonic binding of zinc to extracellular protein domains with nanomolar affinity; (ii) phasic modulation of surface proteins following exocytosis of vesicular zinc; (iii) tonic or phasic modulation of cytosolic proteins in pre- or post-synaptic compartments.

One way to test the cognitive role of synaptic zinc has been to lower its physiological concentration with the use of zinc chelators. As such, local zinc chelation allows assessing the *net* effect of zinc at the expense of not differentiating between potentially overlapping facilitatory and inhibitory effects (Timofeeva and Nadler, 2006), or between the synaptic and extra-synaptic actions of zinc. A number of studies have found zinc chelators to modify hippocampus- or amygdala-dependent behaviors. Thus, intra-hippocampal infusion of the membrane-permeable chelator diethyldithiocarbamate (DEDTC) was shown to impair a delayed matching-to-place version of the water maze task (Frederickson et al., 1990). In this task, rats must remember the last location of a hidden platform submerged in a pool of opaque water. The platform location remains constant between two consecutive runs in a trial, whereas it is changed to a different location between trials separated by 10 min. The shorter latency to find the platform on the second run of a given trial (i.e., savings) indicates a memory for the last location (Steele and Morris, 1999). Because the animal has to update its memory in every trial, a high cognitive load is necessary to perform the task. DEDTC injection (i.e., spreading across all hippocampal subregions) shortly before training selectively suppresses the savings in time normally observed on the second run. Moreover, the memory impairment is transitory and parallels the time course of vesicular zinc chelation, arguing against the possibility of hippocampal damage from the injection. Another study assessed the effect of intra-hippocampal DEDTC injections on the standard version of the Morris water maze, which measures incremental, spatial reference memory (Lassalle et al., 2000). DEDTC during training prevents mice from learning the location of a fixed hidden platform, as they show no spatial bias during the probe test. In contrast, post-training DEDTC injections do not affect memory consolidation or recall. Interestingly, these two studies found that injection of the cell-impermeable zinc chelator CaEDTA fails to replicate the effects of DEDTC. Although initially interpreted as a lack of effect of extracellular zinc on spatial memory, subsequent reports suggested that the high concentrations of CaEDTA that were used (i.e., 200 mM) can also deplete intra-vesicular zinc, perhaps by creating an outward gradient (Frederickson et al., 2002). Hence, the basis for the memory impairment induced by DEDTC, but not CaEDTA, remains unclear. On the other hand, both DEDTC and CaEDTA were found to impair contextual fear conditioning (Daumas et al., 2004). This rapid, one-trial form of associative learning allows better temporal separation between the different phases of memory. Acute zinc chelation impairs the consolidation of contextual memory, but not its recall once the memory has been established. Of note, hippocampal zinc chelation in the same mice does not impair acoustic conditioning, which does not depend on the hippocampus. Together, these studies suggest that reactive zinc, perhaps of synaptic origin, is required for the acquisition or consolidation of hippocampus-dependent memory, depending on the task involved.

A recent study analyzed the role of reactive zinc in the rat amygdala (Takeda et al., 2010). Continuous perfusion of the amygdala with 1 mM CaEDTA during fear conditioning or during recall was found to increase, rather than decrease, the expression of memory. It should be noted that the intense conditioning protocol used here (i.e., >100 foot shocks over 1 h) is more akin to inescapable shock stress models of depression than to associative learning paradigms. Interestingly, this low concentration of CaEDTA elevates the basal extracellular levels of zinc and glutamate by two to fourfold, as well as the probability of vesicular release, suggesting that its potentiating effect may be due to disinhibition of amygdala synapses. The notion that zinc could indirectly modulate memory through effects on excitability is consistent with the predominantly anticonvulsant effects of zinc (Mitchell and Barnes, 1993; Dominguez et al., 2003).

Dietary zinc deficiency has also been used to assess the role of brain zinc in memory. A number of reports have demonstrated behavioral alterations in animals fed a zinc-restricted diet for several weeks, including increased anxiety or depression, and impaired spatial memory and extinction learning (Keller et al., 2000; Takeda et al., 2007; Tassabehji et al., 2008; Whittle et al., 2010). One caveat with dietary zinc deficiency models is that they can lead to multisystemic dysfunction, particularly in peripheral tissues with rapid turnover, and compromise many zinc-dependent reactions (Vallee and Falchuk, 1993). In the hippocampus, zinc deficiency has been shown to reduce the total number of synaptic vesicles in boutons (Lu et al., 2000). Hence, the extent to which behavioral changes associated with zinc deficiency are directly attributable to a reduction in synaptic zinc or even to reactive zinc in neurons is controversial.

To circumvent the lack of subcellular specificity attained by zinc chelation or dietary zinc deficiency, recent studies have analyzed the behavioral phenotype of ZnT-3 mutant (KO) mice. These mice are anatomically normal, but show no zinc staining or enrichment of total zinc in synaptic terminals (Cole et al., 1999; Linkous et al., 2008). In addition, total zinc levels in brain regions normally devoid of vesicular zinc, or seizure-induced increases in somatic (i.e., extra-synaptic) zinc are intact in ZnT-3 KO mice (Lee et al., 2000), indicating that zinc depletion is restricted to synaptic vesicles. These data are consistent with the selective expression of ZnT-3 in synaptic vesicles (Wenzel et al., 1997), and imply that extra-synaptic zinc pools can be regulated independently of vesicular zinc. Behaviorally, ZnT-3 KO mice are characterized by normal sensorimotor function and open field activity (Cole et al., 2001). Anxiety and depressive-like behaviors are also normal in the ZnT-3 KO mice. Interestingly, most of the cognitive deficits identified in ZnT-3 KO mice have been observed in tasks that require disambiguation, detailed attention or reverse learning. Thus, ZnT3 KO mice show normal fear conditioning after repeated delivery of tone-shock pairs (Cole et al., 2001), but display reduced memory

when the tones are made discontinuous or are explicitly unpaired with the shocks (Martel et al., 2010, 2011). One possible explanation for this result is that the latter paradigms are thought to recruit cerebrocortical areas (i.e., zincergic), whereas simple cued conditioning is largely supported by thalamo-amygdala synapses devoid of vesicular zinc (Kodirov et al., 2006). Spatial reference memory following intense training in the Morris water maze task is also preserved in ZnT-3 KO mice (Cole et al., 2001; Martel et al., 2011). However, mutants show a transient learning delay in the reversal phase (i.e., when the platform was relocated to the opposite quadrant). Another study found that ZnT-3 KO mice have a profound deficit in a T-maze non-matching-to-place working memory task, in which mice are rewarded for alternating in quick succession in a two-choice maze (Sindreu et al., 2011). Spatial working memory has been shown to be highly sensitive to hippocampal dysfunction (Deacon and Rawlins, 2006). Because the hippocampus also is indispensable for the formation of detailed contextual memories (Wiltgen et al., 2010), the same group assessed the performance of ZnT-3 KO mice in a contextual discrimination task. Strikingly, ZnT-3 KO mice are unable to differentiate between the conditioning context and a similar context where they have never been shocked (Sindreu et al., 2011). The fact that ZnT-3 KO mice display normal levels of contextual memory in non-discriminative conditioning rules out an impairment of performance that prevented the expression of otherwise intact memory. Moreover, the contextual discrimination deficit in ZnT-3 KO mice can be reproduced by injecting adult wild type mice with the zinc chelator TPEN into the MF-rich CA3 subregion. This latter result points to an acute effect of vesicular zinc in CA3 that was not compensated for in ZnT-3 KO mice. A marginal deficit in social discrimination, which is another form of hippocampus-dependent memory, has also been reported in ZnT-3 KO mice (Martel et al., 2011). Together, the evidence suggests that vesicular zinc is required for some forms of hippocampus- and amygdala-dependent memories, but it does not affect normal general performance.

The neuronal circuits where ZnT-3 is acting to support these different forms of memory are poorly defined. The contribution of specific synaptic pathways to learning and memory is just starting to emerge, and such understanding will be instrumental to fully account for the complex mnemonic phenotype of ZnT-3 KO mice. A useful example comes from the effect of genetically inactivating CA3 neurons (Nakashiba et al., 2008). Although Schaffer-collateral inputs to CA1 are greatly silenced, mice are still capable of acquiring spatial reference memory in the Morris water maze task. This finding not only implies that direct cortical projections to distal CA1 can support incremental learning, but it may also explain why ZnT-3 KO mice perform this task normally, as the entorhinal projection to CA1 is entirely non-zincergic (**Figure 1**). In contrast, the deficits in contextual discrimination and complex tone conditioning in ZnT-3 KO mice may reflect the recruitment of zincergic mossy fibers and cortico-amygdaloid projections during performance of these tasks, respectively.

Another lingering question is why zinc chelators appear to cause stronger behavioral deficits than the genetic removal of vesicular zinc. Given the absence of reactive zinc in vesicles of ZnT-3 KO mice, molecular redundancy in vesicular zinc transport seems unlikely. As discussed earlier, reactive zinc also may be found in other subsynaptic compartments. One possibility, then, is that zinc chelators may remove additional pools of zinc important for behavior that are spared in ZnT-3 KO mice. In keeping with this view, age-related decreases in non-vesicular zinc are observed in mouse hippocampus and correlate with late-onset deficits in spatial reference memory (Adlard et al., 2010). If non-vesicular zinc has any behavioral relevance, one would predict that zinc chelators aggravate the memory deficits of ZnT-3 KO mice. We know of no such experiments from the literature. One pool of non-vesicular zinc that potentially may participate in memory is the cytosolic zinc bound to MT-3. However, neither deletion nor over-expression of MT-3 alter passive avoidance, spatial reference memory or reversal learning (Erickson et al., 1997). An alternative view, not necessarily incompatible with the above, is that mice compensate, at least in part, for the absence of vesicular zinc during development. Although no compensatory mechanism has been reported in ZnT-3 KO mice so far, rescue experiments in adult subjects or conditional deletion of ZnT-3 will be necessary before compensation can be formally ruled out. Finally, the development of cell-impermeable chelators with faster rates of zinc chelation and improved ion selectivity would help draw more direct comparisons between genetic and pharmacological manipulations of zinc on behavior.

SYNAPTIC ZINC AND NEURONAL SIGNALING

SYNAPTIC TRANSMISSION AND PLASTICITY

The synaptic accumulation of reactive zinc and its involvement in several forms of memory suggest a role(s) for zinc in synaptic physiology. Zinc chelators or ZnT-3 deletion do not alter fast excitatory, AMPAR-mediated synaptic transmission evoked by low-frequency stimulation in brain slices (Li et al., 2001a; Lopantsev et al., 2003; Kodirov et al., 2006; Mott et al., 2008; Pan et al., 2011). However, a number of synaptic receptors are known to contain binding sites with nanomolar affinity for extracellular zinc, raising the possibility that zinc modulates their activity in a tonic or phasic manner. In keeping with this, chelation of extracellular zinc increases $GABA_AR$-, NMDAR-, and kainate receptor-mediated currents at MF synapses (Vogt et al., 2000; Molnar and Nadler, 2001; Ruiz et al., 2004; Mott et al., 2008), potentiates ASIC-mediated currents in cortical neurons (Chu et al., 2004), and reduces glycinergic currents in hypoglossal neurons of the brainstem (Hirzel et al., 2006). Zinc inhibition of NMDARs has been greatly examined in heterologous expression systems and found to operate via two mechanisms: one that is low-affinity and voltage-dependent, and another one that is high-affinity and voltage independent (Paoletti et al., 1997; Traynelis et al., 1998; Choi and Lipton, 1999). Importantly, knock-in mice with point mutations previously implicated in zinc binding to the α1 glycine receptor 1 subunit (Hirzel et al., 2006) or the GluN2A NMDAR subunit (Nozaki et al., 2011) display sensorimotor deficits and impaired pain processing, respectively. Likewise, high-affinity zinc inhibition of $Ca_v3.2$ channels may limit hyperalgesia in peripheral neurons (Nelson et al., 2007). These findings highlight the importance of zinc modulation of ion channels in clinically relevant responses. However, few of the above studies have examined the origin of the zinc that binds to these ion channels. Using glutamate iontophoresis in CA3 neurons, it has been shown that the

increase in NMDAR currents caused by zinc chelators is abolished in ZnT-3 KO mice and is occluded by an increase of similar magnitude triggered by briefly blocking pre-synaptic exocytosis (Vogt et al., 2000). Analogously, zinc chelation no longer enhances kainate receptors in *mocha* mice with reduced vesicular zinc (Mott et al., 2008). These data indicate that, at least at MFs, tonic vesicular zinc release modulates post-synaptic receptors. At variance with this conclusion, post-synaptic NMDAR currents were found to be of similar amplitude in ZnT-3 KO and control CA3 neurons, although in this case the differential effect of zinc chelation between genotypes or the presence of contaminant zinc was not considered (Lopantsev et al., 2003). Perhaps more importantly, little is known as to the impact of extracellular zinc binding to synaptic ion channels on mnemonic processes or neuronal plasticity. The recent development of GluN2A knock-in mice (Nozaki et al., 2011), which express NMDARs insensitive to nanomolar zinc (H128S substitution) but are presumably still inhibited by higher zinc concentrations, will help establish the role of high-affinity zinc binding on NMDAR-dependent forms of learning and synaptic plasticity.

Ideally, one would like to know the effect of synaptic zinc on synaptic responses elicited by the behavior of interest or to the sort of spike patterns associated with that behavior. This information is not yet available at the electrophysiological level. Converging data from three types of zincergic synapses (the MF-to-CA3, the CA3-to-CA1, and the auditory cortex-to-lateral amygdala synapse) indicate that endogenous zinc is required for the induction of long-term potentiation (i.e., LTP) of AMPA currents, a candidate cellular correlate of learning. Whereas post-synaptic mechanisms have been strongly implicated in the expression of LTP at CA3-to-CA1 synapses, MF-to-CA3 plasticity appears to largely rely on pre-synaptic increases in release probability (Nicoll and Schmitz, 2005). Thus, Lu et al. (2000) showed that the cell-permeable zinc chelators dithizone and DEDTC reversibly block MF LTP. Along these lines, CaEDTA (7.5–10 mM) was also shown to block the induction of MF LTP by two other laboratories (Li et al., 2001a; Huang et al., 2008). Notably, lower CaEDTA concentrations (1–2 mM) or partial (∼50%) removal of vesicular zinc by mutation of the AP-3 δ subunit (Seong et al., 2005) fail to inhibit MF LTP (Lu et al., 2000; Vogt et al., 2000; Li et al., 2001a). In contrast, low concentrations of CaEDTA (1–2 mM) or TPEN (0.1 μM) suffice to inhibit LTP at CA1 and lateral amygdala synapses (Izumi et al., 2006; Kodirov et al., 2006; Takeda et al., 2009). The surprising observation here is that chelator-induced LTP block appears to be complete in CA1 and amygdala, even though only a fraction of their synapses react for vesicular zinc. Paralleling the level of zinc chelation required to affect LTP, application of a high zinc concentration (50–100 μM) is needed to potentiate MF synapses (Li et al., 2001a; Huang et al., 2008), whereas a lower zinc concentration (1–10 μM) enhances LTP in CA1 (Izumi et al., 2006; Takeda et al., 2009; Lorca et al., 2011). Importantly, none of the above studies observed an effect of zinc chelation on basal transmission, paired-pulse modulation, or LTP maintenance, suggesting a role for synaptic zinc in the induction of long-term plasticity. Despite this emerging consensus, not all data are concordant, and others have failed to block MF LTP when using TPEN (20–100 μM; Matias et al., 2006). Collectively, it could be argued that the differential sensitivity to zinc manipulation among synapses grossly correlates with the amount of vesicular zinc that they accumulate. So what, then, is the effect of completely (and selectively) removing vesicular zinc on synaptic plasticity? Surprisingly, only one recent study examined long-term synaptic plasticity in ZnT-3 KO mice, and obtained both confirmatory and unexpected results (Pan et al., 2011). On one hand, the increase in release probability normally observed following tetanus-induced MF LTP was absent in ZnT3 KO mice, confirming a role for vesicular zinc in pre-synaptic plasticity. However, MF LTP was still observed in ZnT3 KO mice, and this was found to be due to disinhibiton of a separate, post-synaptic calcium-dependent mechanism of MF LTP that is normally blocked by zinc in wild type mice (Pan et al., 2011). Thus, in the presence of vesicular zinc, MF LTP is expressed via a pre-synaptic mechanism, but when vesicular zinc is removed, a post-synaptic mechanism is recruited instead, indicating a dual role for zinc. Similar conclusions were reached when using a new, fast-acting extracellular zinc chelator, ZX1. Importantly, both pre- and post-synaptic components of MF LTP were observed in the presence of NMDAR antagonists, differentiating them from a recently described form of MF LTP affecting NMDAR currents (Kwon and Castillo, 2008; Rebola et al., 2008). LTP at other synapses in ZnT3 KO mice remains to be examined. Regarding synaptic plasticity in ZnT3 KO mice, two reported observations require clarification: first, and in contrast to ZX1-perfused wild type mice, post-synaptic calcium chelation does not fully block MF LTP in ZnT3 KO mice; secondly, a basal increase in the frequency of spontaneous MF glutamate release in ZnT3 KO mice has been shown (Lopantsev et al., 2003; Pan et al., 2011). Inducible downregulation of ZnT3 in adult brain will help determine whether these differences reflect homeostatic compensations to gene deletion.

Conclusions on the effects of CaEDTA in LTP are somewhat confounded by its properties and should be confirmed in the future with more selective extracellular zinc chelators, such as Tricine (Paoletti et al., 2009) or ZX1. Given the high-affinity constant of CaEDTA for zinc ($K_D = 10^{-16}$), its concentration-dependent effects have been attributed to the slow kinetics of zinc chelation, which are largely dictated by the slow off-rate for calcium. Thus, low CaEDTA would remove extracellular zinc at equilibrium but it would fail to intercept much of synaptically released zinc. On the other hand, concentrations of CaEDTA higher than 5 mM (i.e., those that block MF LTP) have been shown to reduce the vesicular zinc content under steady-state conditions (Lavoie et al., 2007; Ketterman and Li, 2008). As CaEDTA is negatively charged at physiological pH and hence unlikely to cross the plasma membrane, the intracellular zinc reduction may reflect a homeostatic response to extracellular chelation of zinc or other cations. It is therefore unclear if high CaEDTA blocks MF LTP by chelating zinc before or following its actual release. The extracellular specificity of alternative zinc chelators may be assessed by quantifying the extent of vesicular zinc depletion when blocking pre-synaptic exocytosis in the presence of the chelator. Future studies should also address the importance of intracellular zinc chelation in pre- and post-synaptic compartments on LTP.

SYNAPTIC SIGNAL TRANSDUCTION

Both learning and repetitive synaptic activation stimulate intracellular signal transduction cascades necessary for memory formation and synaptic plasticity. The involvement of synaptic zinc in some forms of memory and LTP hence suggests that zinc may participate in the mechanisms of neuronal signal transduction. Indeed, three independent groups have recently implicated endogenous zinc in the activation of intracellular signal cascades at MFs (**Figure 3**; Huang et al., 2008; Besser et al., 2009; Chorin et al., 2011; Sindreu et al., 2011). MF tetanization or direct zinc application (100 μM) in the presence of NMDAR antagonists induces a form of LTP that requires TrkB activity, and is abrogated by CaEDTA (Huang et al., 2008). Whether this type of LTP requires a cAMP-dependent increase in the probability of glutamate release as in classic MF LTP_{AMPA} has not been shown yet. Providing a possible mechanism, zinc application to cultured neurons activates the cytosolic tyrosine kinase Src, increasing phospho-activation of the TrkB receptor in the absence of its ligand BDNF. It had been previously suggested that zinc activation of TrkB operates through the conversion of pro-BDNF to mature BDNF (Hwang et al., 2005). However, the fact that zinc still potentiates MFs in BDNF KO mice suggests that such mechanism might not be essential. Downstream signals, including the Erk1/2 MAP kinase and PLCγ1 pathways, are also activated by zinc in cultures. Although it remains to be shown that synaptic zinc activates Src–TrkB signaling during induction of this form of MF LTP, the data provide circumstantial evidence that this may be the case. TrkB activation is most prominent in axon terminals (Spencer-Segal et al., 2011), whereas the Src kinase accumulates in NMDAR complexes (Salter and Kalia, 2004). Hence, the pre- or post-synaptic MF localization of this pathway and the route(s) of zinc entry remain to be clarified. Since primary neuron cultures typically fail to stain for vesicular zinc (Li et al., 2003; Love et al., 2005; Grabrucker et al., 2011), these issues may need to be addressed in more intact preparations.

Exogenous zinc application (100 μM) has also been reported to increase phosphorylation of Erk1/2 in CA3 somata of young (P8-P16) mice, an effect that is prevented by the Gαq inhibitor YM-254890 (Besser et al., 2009). Interestingly, brief MF tetanization (10 pulses at 66 Hz) triggers a Gq–dependent post-synaptic calcium increase that is reduced by half in ZnT-3 KO mice, suggesting that synaptic zinc can contribute to G protein-coupled receptor (GPCR) signaling even at a developmental age when synaptic vesicles are only partially stocked with zinc (Frederickson et al., 2006a). Furthermore, zinc-induced Gq signaling leads to rapid Erk-dependent up-regulation of KCC2, a K^+/Cl^- co-transporter, increasing the transmembrane Cl^- gradient in CA3 neurons (Chorin et al., 2011). Because KCC2 up-regulation is absent in GPR39 KO mice, this orphan receptor might mediate the metabotropic actions of synaptic zinc. Consistent with this, GPR39 contains extracellular domains that coordinate zinc (Popovics and Stewart, 2011). Interestingly, Erk1/2 activation via TrkB also up-regulates KCC2 as long as PLCγ1 is not co-activated (Rivera et al., 2004). Future studies may clarify the significance of these converging signals to adult synaptic plasticity or memory formation. Intriguingly, similar tetanization regimes and molecular determinants have been associated with the induction of MF LTP_{NMDA} (Kwon and Castillo, 2008; Rebola et al., 2008). In this regard, another study showed that MF stimulation at 50 Hz triggers a form of short-term plasticity (∼10 min-long) that activates, and is modulated by, the pre-synaptic Erk1/2-Synapsin pathway (Vara et al., 2009). Synapsin is a protein that tethers synaptic vesicles to the actin cytoskeleton to modulate transmitter release (Fdez and Hilfiker, 2006), and shown to interact with the plasticity-related protein Rab3A (Giovedi et al., 2004). Suggesting some behavioral relevance for the above observations, dentate gyrus granule cells can fire in bursts of up to 50 Hz when the animal visits a location corresponding to the place field center of the cell (Jung and Mcnaughton, 1993). More recently, it was found that contextual conditioning induces the pre-synaptic activation of the Erk1/2-Synapsin I pathway at MFs (Sindreu et al., 2011). Furthermore, training-induced activation of Erk1/2 is selectively ablated in MFs of ZnT-3 KO mice; partial inhibition of Erk1/2 restricted to granule cells causes a similar deficit in contextual discrimination as that observed in ZnT-3 KO mice. These data represent the first direct evidence that vesicular zinc regulates neuronal signal transduction associated with memory formation. Mechanistically, ZnT-3 KO mice show disinhibition of MAPK tyrosine phosphatases, suggesting that it may underlie impaired Erk activation. Consistent with this, the authors showed that activity-dependent zinc release couples to phosphatase inhibition and Erk1/2 stimulation at MF terminals. The inhibition of MAPK phosphatases by zinc is in keeping with previous observations in neuron cultures (Ho et al., 2008). Because direct Erk1/2 inhibition has been shown to completely block glutamate release (Jovanovic et al., 2000), zinc inhibition of MAPK phosphatases may regulate other pre-synaptic proteins in addition to Synapsin I. At present, it is unclear how zinc may be recycled back into the pre-synaptic terminal to inhibit phosphatases. The identity of the zinc-sensitive MAPK phosphatase also remains to be shown. Suggesting an important role for tyrosine phosphatases in Erk1/2 regulation, catalyzed dephosphorylation of the tyrosine residue (Y185) appears to be the initial and rate-limiting step in Erk1/2 inactivation (Zhou et al., 2002). Previous studies indicated that zinc can inhibit tyrosine phosphatases with high-affinity by binding to their conserved catalytic domain (Brautigan et al., 1981; Haase and Maret, 2003; Redman et al., 2009). How, then, synaptic zinc regulates select signaling pathways via phosphatase inhibition is an intriguing question.

The aggregate evidence indicates that synaptic zinc is a positive regulator of Erk1/2 at MF synapses, activating it through one or more mechanisms (**Figure 3**). Whereas zinc-dependent Gq signaling may not require zinc influx, released zinc would need to gain access into the cytosol regulate Src or MAPK phosphatases. At MFs, voltage-gated calcium channels represent the main route for pre- and post-synaptic entry of divalent cations (Reid et al., 2001), and calcium channel inhibition suppresses pre-synaptic Erk1/2 at MFs (Sindreu et al., 2011). In addition, currently undefined zinc importers or co-transporters could promote (re-)uptake of extracellular zinc. Whether, similarly to Erk1/2, activation of TrkB or Gq signaling specifically in granule cells or CA3 neurons supports hippocampus-dependent memory is still not known. Also, the possibility of cross-talk between these zinc-regulated pathways has not been examined. For instance, although basal TrkB activation is normal in ZnT3 KO mice (Sindreu et al., 2011), it is unclear if TrkB further stimulates Erk1/2 during learning. Conclusively

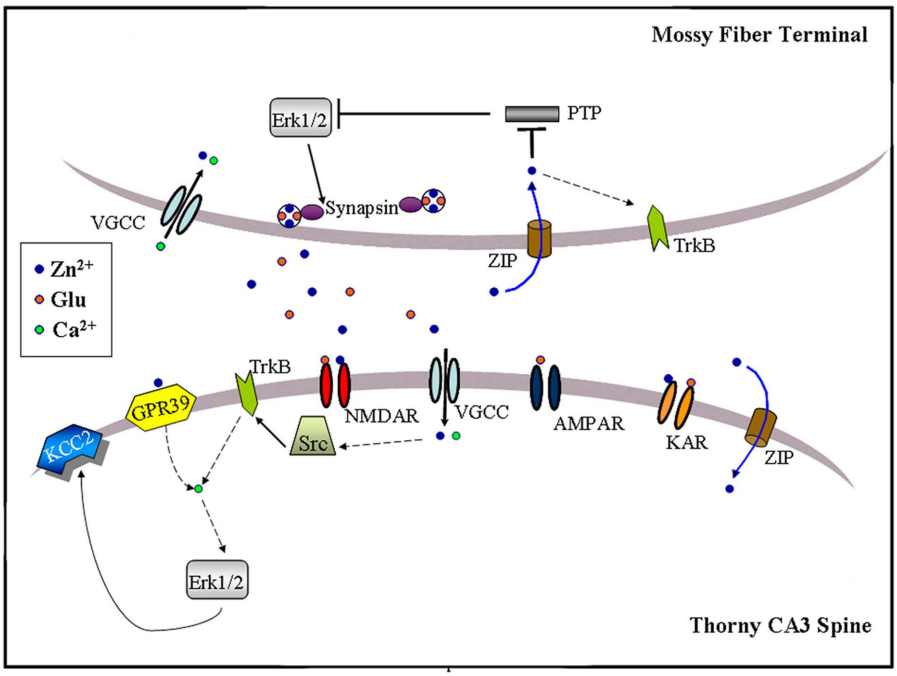

FIGURE 3 | Schematic illustration of zinc-activated signal transduction pathways at the MF-to-CA3 pyramid synapse. Synaptic zinc may activate three signaling pathways: (1) Trans-activation of TrkB receptors at either pre- or post-synaptic sites, perhaps through Src kinase and leading to downstream activation of Erk1/2, Akt or PLCγ; (2) Post-synaptic activation of a GPR39, leading to IP3-dependent intracellular calcium release and KCC2 up-regulation, possibly via Erk1/2 activation; (3) Pre-synaptic Erk1/2 activation, perhaps via inhibition of a MAPK tyrosine phosphatase, leading to synapsin I phosphorylation. Synaptically released zinc may cross the plasma membrane via calcium-permeable channels (VGCC) or dedicated zinc transporter mechanisms (ZIP). Extracellular zinc may also inhibit surface NMDAR and kainate receptors (KAR). Dashed arrows indicate the presence of intermediate steps between connected elements.

establishing the pre- or post-synaptic localization of these various pathways will help draw connections among them. Mechanistically, zinc-activated intracellular cascades will need to be put in context with the previous molecules implicated in MF plasticity, such as the adenylyl cyclases and their downstream targets (Nicoll and Schmitz, 2005). A large body of literature indicates that the Erk1/2 pathway (i.e., Ras–b–Raf–MEK–Erk) acts as a node integrating several upstream signals to control memory and neuronal plasticity, mainly through actions on dendritic proteins and gene transcription (Impey et al., 1999; Thomas and Huganir, 2004; Ye and Carew, 2010). However, there is also evidence that Erk1/2 affects cognition via pre-synaptic mechanisms (Kushner et al., 2005; Cui et al., 2008). Circumstantially, zinc can have opposite effects on MAPK in other cellular models. For instance, zinc can decrease Erk signaling in oocytes by inhibiting Raf, an effect that may be counteracted by ZnT-1 (Jirakulaporn and Muslin, 2004). It remains to be explored if reactive zinc can bidirectionally modulate Erk signaling by engaging various mechanisms in neurons.

A pertinent question is to what extent the effects of synaptic zinc on ion channels and signal transduction cascades are functionally related, and whether they co-occur at individual synapses. At MFs, the effects of zinc on Gq-, TrkB-, and Erk-dependent signaling are all spared by NMDAR blockers, in line with the ability of this synapse to express NMDAR-independent forms of plasticity. Moreover, the effects of synaptic zinc on Erk1/2 activation or TrkB-dependent plasticity are not reproduced at CA1 synapses (Huang et al., 2008; Sindreu et al., 2011), possibly as a result of molecular heterogeneity between synapses. In contrast, blockade of CA1 LTP by zinc chelators has been attributed to the untimely activation (due to disinhibition) of NMDARs upstream of calcineurin, nitric oxide, and p38 (Izumi et al., 2006, 2008). Whether NMDAR-dependent forms of plasticity activate other downstream signals that regulate, or are regulated by, reactive zinc is unclear. CaMKII, PKA, PKC, or Akt are normally activated in ZnT-3 KO hippocampus (Sindreu et al., 2011). However, *in vitro* studies have shown that zinc unbinding from PKC can trigger the activation of this kinase (Knapp and Klann, 2000; Korichneva et al., 2002), and that constitutively active PKC is, in turn, sufficient to increase the expression of genes containing metal regulatory elements (Aras et al., 2009). Thus, PKC activation during synaptic plasticity could potentially trigger intracellular zinc signaling. The effect of acutely chelating reactive zinc from brain tissue on these pathways remains to be shown. On the other hand, block of LTP at cortico-amygdala synapses by zinc chelators can be rescued by $GABA_AR$ antagonists (Kodirov et al., 2006), implying that zinc can gate LTP via heterosynaptic inhibition of feed-forward interneurons. The authors proposed that such a mechanism may provide spatial control of amygdala LTP between cortical and thalamic afferents.

Future experiments examining how these, and other, mechanisms of zinc action affect cognition are warranted. The deficits

in synaptic plasticity and biochemical activation induced by zinc blockade are in agreement with the memory impairments observed in ZnT-3 KO mice or after zinc chelation. While the ZnT-3 KO mouse model has now provided strong evidence for a role of vesicular zinc in cognition, circumstantial evidence suggests that other sources of reactive zinc may also be involved. Altogether, the data we have discussed here lend further support to the hypothesis that synaptic zinc may promote learning and memory through actions on neuronal signal transduction. Traditionally viewed as an inhibitor of glutamatergic transmission, synaptic zinc may turn out to play complementary roles with glutamate in the service of memory and neuronal plasticity: zinc may recruit additional plasticity mechanisms during increased neuronal firing associated with learning, prevent tonic interference from other signaling pathways, or select between converging synaptic contacts to promote memory formation. Many of the recent findings on synaptic zinc had not been anticipated only 5 years ago. Should this trajectory continue, the next few years will reveal interesting new facets of zinc signaling in the brain.

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Ras–ERK signaling in behavior: old questions and new perspectives

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The role of Ras–ERK signaling in behavioral plasticity is well established. Inhibition studies using the blood–brain barrier permeable drug SL327 have conclusively demonstrated that this neuronal cell signaling cascade is a crucial component of the synaptic machinery implicated in the formation of various forms of long-term memory, from spatial learning to fear and operant conditioning. However, abnormal Ras–ERK signaling has also been linked to a number of neuropsychiatric conditions, including mental retardation syndromes ("RASopathies"), drug addiction, and L-DOPA induced dyskinesia (LID). The work recently done on these brain disorders has pointed to previously underappreciated roles of Ras–ERK in specific subsets of neurons, like GABAergic interneurons of the hippocampus or the cortex, as well as in the medium spiny neurons of the striatum. Here we will highlight the open questions related to Ras–ERK signaling in these behavioral manifestations and propose crucial experiments for the future.

Keywords: Ras, ERK, neurofibromin, ERK1, drug addiction, L-DOPA induced dyskinesia, RASopathies, Ras-GRF1

Since the discovery, 15 years ago, of the role of Ras–ERK signaling in long-term potentiation first (English and Sweatt, 1996, 1997) and then in memory formation (Brambilla et al., 1997; Silva et al., 1997; Atkins et al., 1998), a considerable experimental evidence has been accumulated to support the idea that this signal transduction pathway is crucial for strengthening synaptic connections in a variety of behavioral processes. The scope of this work is not to provide an extensive review of the available data but rather to highlight the aspects which still need to be clarified in order to gain a comprehensive description of the molecular processes involving Ras–ERK in behavior. For a detailed analysis of the published evidence, we refer to a few excellent review articles appeared in the last 4–5 years (Davis and Laroche, 2006; Girault et al., 2007; Santini et al., 2008; Samuels et al., 2009).

THE RAS–ERK PARADOX: BALANCING SYNAPTIC EXCITATION AND INHIBITION

Early on, when the first two publications on genetically altered mice in the Ras–ERK pathway became simultaneously available, what we call here the Ras–ERK paradox appeared immediately clear: any manipulation of this pathway either causing an enhancement or a partial inhibition of cell signaling, would lead to learning and memory deficits (Brambilla et al., 1997; Silva et al., 1997). In fact, not only the ablation from the mouse of Ras-GRF1, a CNS specific neuronal activator of Ras proteins, results in memory deficits but also the gene disruption of the neurofibromatosis type 1 (NF1) gene, the locus responsible for the expression of the Ras-specific negative regulator *neurofibromin*, a GTPase activating protein, causes significant learning impairments. Although these early studies did not provide any biochemical evidence supporting the expected contrasting cell signaling effect of the two mutations (Ras-GRF1 loss would attenuate Ras activity while neurofibromin should enhance it), these genetic results initially suggested that any bidirectional alteration from the "physiological" neuronal ERK activity would negatively impact on the brain's ability to correctly process information. There are many example in which biological responses follow an "inverted U" shape, for instance the effect on memory mediated by corticosteroids, but the Ras–ERK paradox is a unique case in which the modulation of an intracellular signal transduction pathway results in such a clear detrimental effect on cognitive functions. This observation was clearly underappreciated at that time, but in retrospect it was and it is still a formidable obstacle to the development of cognitive enhancers which, either directly or indirectly, would impact on ERK signaling. One of the reasons why this question was not promptly investigated is that immediately after the Ras-GRF1 and NF1 KO publications, SL327 became available. This small chemical inhibitor of MEK1/2, the kinases upstream of ERK1/2, has been extremely useful in dissecting the effect of ERK inhibition on behavior since it rapidly passes the brain–blood barrier. Later on, other similar drugs started to be used, e.g., PD184161, with similar effects. Predictably, ERK inhibition does block long-term experience- and drug-dependent behavioral plasticity (see the above articles for review). In addition, this pharmacological approach has also been instrumental in demonstrating a central role of the ERK pathway in psychiatric conditions less dependent on memory functions such as chronic stress and depression (see for instance Duman et al., 2007). However, this plethora of inhibition studies, generated in the last decade, has not helped us much in resolving the ERK paradox but has simply confirmed that this pathway is a necessary ("permissive") condition to cause long-term changes behavior. Once again, the use of genetically altered animals has instead significantly contributed to define a much more complex role of Ras–ERK in the brain. In 2002 the initial

hypothesis that a general and "non-cell-specific" increase in ERK signaling may lead to memory impairments had to be dismissed on the basis of two publications. Firstly, the ideal system to test this hypothesis has been represented by the ERK1 mutant mice which show a general enhancement of ERK activity in the brain (Mazzucchelli et al., 2002). This is due to a de-repression of ERK2, the main MAP kinase. In normal conditions, ERK1 acts as a built-in partial agonist, keeping ERK2 activity tightly regulated (Vantaggiato et al., 2006; Indrigo et al., 2010). Despite this general effect of the biochemistry of ERK2, ERK1 mutant animals do not show any sign of cognitive deterioration as one would have predicted from the early studies on the NF1 KO mice. On the contrary, ERK1 deficient mice manifest specific memory enhancing effects, including striatum-dependent operant conditioning long-term memory formation, increased fear memory consolidation and extinction, novel object recognition memory, and cocaine-dependent associative and non-associative learning but not spatial memory changes (Mazzucchelli et al., 2002; Cestari et al., 2006; Ferguson et al., 2006; Tronson et al., 2008; Berardi et al., 2011). Interestingly, while two independently generated Ras-GRF1 KO lines seem to show behavioral responses which are the mirror image of those found in the ERK1 mutants, a mutant showing a mild over-expression of Ras-GRF1 manifest a similar memory enhancing effect in most tasks but not, quite surprisingly, in spatial memory ones (Fasano et al., 2009a; Berardi et al., 2011; D'Isa et al., 2011). Thus, the results obtained with the analysis of the ERK1 mutants indicate that a general enhancement of Ras–ERK signaling can promote learning and memory functions. However, this observation is still in apparent contrast to the phenotype observed in the NF1 mice in which, supposedly, a general ERK enhancement occurs as well. The explanation of this discrepancy was provided by two papers in 2002 and in 2008, both from the Silva's lab. These two papers radically changed our view on cell signaling mediated by Ras–ERK in the brain since they clearly demonstrated that the Ras-dependent effect of *neurofibromin* ablation is specifically linked to an increase in GABA inhibition which is the likely cause of the learning and memory deficits. Either using the global heterozygous NF1 mice or cell-specific promoters to drive CRE expression in conditional NF1 mutants, Silva and co-workers showed that the behavioral effect was compatible with an enhancement of Ras activity in GABAergic interneurons that in turn may affect synaptic plasticity in key areas of the brain implicated in learning, such as the hippocampus. Interestingly, the memory deficits could be partially rescued by either reducing (pharmacologically or genetically) Ras activity (especially of the K-Ras isoform) or by partially inhibiting GABA activity using receptor antagonists. These experimental observations are important since they provide a likely explanation why loss of *neurofibromin* in NF1 patients may lead to learning disabilities. Importantly, they may also provide a rationale to understand other genetic diseases characterized by gain of function mutations in the Ras–ERK pathway leading to mental retardation, the now called RASopathies or Ras–MAPK syndromes (Aoki et al., 2008; Tidyman and Rauen, 2009). The clinical relevance of these disorders is such that a cogent question arising from the work on NF1 mutant mice requires an urgent answer: why do these changes in the Ras–ERK activity only occur in GABAergic cells and not in all neurons? Indeed the data provided by the Silva's 2008 paper indicate that a mutation restricted to glutamatergic pyramidal cells, using the CamKII promoter-CRE line has no impact on behavior. Consistently, our own data on a conditional K-Ras G12V knock-in mutant line also show that a pyramidal specific activation of this Ras isoform has no effect on learning and memory (Papale et al., 2010). On the contrary, the CRE line driving expression under the Synapsin I early promoter leads to significant learning impairments in both NF1 and K-Ras12V mutants. In order to explain these observations, some possibilities arise. Firstly, expression analysis provided by the Allen Brain Atlas (www.brain-map.org) of both NF1 and K-Ras genes seems to indicate that their transcriptome levels are higher from late embryonic development (E18.5) to early post-natal stages (P4), a phase in which the Synapsin I early promoter is already active while the CamKII promoter is not. In addition, since during the post-natal maturation of GABAergic cells, the expression of NF1 and of K-Ras is maximal while later during pyramidal cell development is down to lower levels the occurrence an increased inhibition would be favored. Indeed, an indirect evidence that K-Ras is acting early in development during a temporal window which coincides with GABAergic development comes from the transgenic mouse model in which the H-RasG12V mutation is expressed under the CamKII promoter. In that case a forced over-expression in pyramidal neurons of this gain of function mutant leads to memory and plasticity improvements (Kushner et al., 2005; Kaneko et al., 2010). Whether this positive effect on memory is specific to this Ras isoform still needs to be seen but it is unlikely. Indeed, to complicate the matter, a recently described targeted expression of the very same H-RasG12V via homologous recombination (gene knock-in) results in significant behavioral impairments which partially recapitulate the phenotypes observed in patients affected by the Costello Syndrome, one of the RASopathies (Viosca et al., 2009). Thus, all available data point to the importance of "when" and "where": if Ras–ERK signaling is predominantly active in the GABAergic compartment, then plasticity and memory impairments may occur while the opposite is true when ERK activity is mainly activated in pyramidal cells. Indeed the real scenario is probably even more complex than that if we take into consideration the global ERK1 KO model in which memory improves despite gene ablation is not cell-type selective and the manipulation alters a protein hardly regulated in development. In this specific case, a possible explanation is that ERK1 loss causes a differential effect between excitation and inhibition by favoring the former. If that is the real case still remain to be seen since appropriated experiments, either using conditional ERK1 mutants or viral-mediated cell-specific gene knockdowns of ERK1, have not yet been performed. However, one prediction which can already be made is that a specific enhancement of ERK2 activity in GABAergic cells via ERK1 ablation should recapitulate the NF1 KO phenotype by bypassing the requirement for an elevated Ras activity. Certainly, this prediction could be incorrect in the case that other Ras-dependent signaling pathways, e.g., the PI3 Kinase cascade, significantly contribute to the mental retardation phenotype observed in the NF1 mutants.

One last comment is necessary on the exact mechanism by which *neurofibromin* and K-Ras cause the enhancement of GABAergic activity. Currently this is unclear but the possible explanations, not mutually exclusive are: (i) enhanced GABA

release; (ii) larger GABA synapses; (iii) more GABA synapses; (iv) enhanced GABA-mediated post synaptic signaling. In any case, it is clear that in cortical regions and in the hippocampus, future experiments targeting the Ras–ERK signaling in a cell-specific manner and at different stages of development will provide substantial new evidence and will tell us whether the development of general cognitive enhancers based on ERK manipulation will ever be a viable therapeutic option for memory impairments.

NEURONAL CELL SIGNALING BEYOND THE RAS–ERK PARADOX: THE STRANGE CASE OF THE STRIATUM

Most cortical and hippocampal regions function by a tight integration of excitatory and inhibitory signals. Alterations of this balance, as we have seen above, may result in either a memory gain or a memory loss. However, not all brain areas work in this way. For instance, the striatum, the input nucleus of the basal ganglia system, is essentially constituted (>95%) by GABAergic projection neurons, the so called medium spiny neurons (MSN; Kreitzer and Malenka, 2008). Instead of generating an output activity toward the thalamus and the motor cortex by balancing excitation and inhibition within its structure, the dorsal portion of the striatum (the ventral one, the nucleus accumbens, may be slightly different) integrates two main neurotransmitter signals, the glutamatergic and the dopaminergic ones, and conveys their action on two distinct subclasses of MSN: the direct pathway neurons, mainly expressing dopamine D1 receptors, and the indirect pathway neurons, mainly expressing D2 receptors. In normal conditions, a balanced activation of both pathways leads to an efficient activation of the thalamus and of the cortex. However, in certain neuropsychiatric diseases, one of the two pathways tends to dominate: an enhancement of the direct pathway leads to motor activation while that of the indirect pathway results in motor inhibition.

Not surprisingly, in recent years, the role of the Ras–ERK pathway and downstream gene expression has extensively been investigated in the striatum, using both pharmacological (e.g., SL327) or genetic approaches. The scenario which has resulted from these studies is that in both the behavioral responses to drugs of abuse ("drug addiction") and in a pathological condition resembling L-DOPA induced dyskinesia (LID) in Parkinson's Disease, an aberrant hyperactivation of Ras–ERK appears to be a key pathogenetic factor (Murer and Moratalla, 2011). In general terms, the most widely studied drugs of abuse, psychostimulants (e.g., cocaine and amphetamine), opiates (e.g., morphine and heroine), and cannabinoids (delta-9-tetrahydrocannabinol, THC) can cause both short-term changes of motor activity or long-term behavioral changes, which can include an enhanced locomotor activity or increased reward responses, as measured in conditioned place preference (CPP) or in self-administration procedures. The fact that pharmacological inhibition of ERK blocks these responses is now well established but, for the same reasons of the memory studies discussed in the previous chapter, it provides little additional information beyond establishing a "permissive" role of this pathway in the process.

On the contrary, mouse models of specific genes in the pathway have significantly contributed to outline an interesting scenario. For instance, the original report on ERK1 KO mice that demonstrated an "instructive" role of ERK signaling in memory consolidation, i.e., these mice showed better striatum-dependent memory, also showed that CPP responses to morphine can be significantly enhanced (Mazzucchelli et al., 2002). This effect is not drug-specific since the same can be seen with cocaine (Ferguson et al., 2006). Similar drug-dependent enhancing effects have subsequently been seen in other mutant mice, most notably a striatal-specific dominant negative form of the transcription factor CREB ("killer CREB") and an overexpressing line for Ras-GRF1 (Fasano et al., 2009a,b). The case of CREB is particularly intriguing since the same dominant negative mutant, not only causes memory impairment when expressed in a "hippocampus" specific mouse line but also leads to instrumental learning deficits and LTP/LTD loss in a striatal-specific line (Pittenger et al., 2002, 2006). Thus, the manipulation of CREB within a given brain area, the dorsal striatum, shows a stimulus-specific effect which is in contrast to what was observed for other mutations in the ERK pathway, most notably Ras-GRF1, in which the gene disruption causes both memory loss and a reduced response to drugs (Brambilla et al., 1997; Fasano et al., 2009a; D'Isa et al., 2011). In the case of CREB, a likely possibility is that a repeated administration of a drug of abuse may lead to a compensatory upregulation of endogenous CREB, as seen previously for hypomorphic mutations of the gene itself or after viral-mediated expression of either WT or other dominant negative mutants of CREB in the Nucleus Accumbens (see Carlezon et al., 2005). It is obvious that more sophisticated experiments will be required to fully understand the role of CREB in striatum specific behavioral plasticity, including its expression specifically in either the direct or in the indirect pathway MSN.

In that respect, the use of BAC transgenic mice expressing the green fluorescent protein (GFP) in the two MSN compartments has enormously facilitated the analysis of the activation profile of the Ras–ERK pathway. Drugs like cocaine specifically activate ERK1/2 in the direct pathway MSN (Girault et al., 2007; Bertran-Gonzalez et al., 2008). On the contrary, antipsychotics like haloperidol, uniquely induced ERK1/2 activity in the indirect pathway. Consistently with the molecular data, a specific activation of the direct pathway leads to motor activation whereas motor inhibition is seen when the indirect pathway is induced.

L-DOPA induced dyskinesia is a severe condition in which chronic administration (several years) of the gold standard treatment of Parkinson's Disease results in abnormal involuntary movements (AIM). This pathological condition can be modeled in rodents by causing an unilateral loss of the substantia nigra pars compacta (SNc) neurons with the neurotoxin 6-hydroxy dopamine (6-OHDA) followed by repeated L-DOPA injections. LID takes several years to appear in patients while in rodents the effect is almost immediate, after one or few injections of the therapeutic drug. In recent years, we have started to understand the molecular mechanisms underlying LID in rodents and the key event occurring in the dorsal portion of the striatum, the target region of the dopaminergic SNc cells, is the supersensitization of D1 receptor signaling through the upregulation of G_{olf} (Girault et al., 2007). In other words, LID is essentially characterized by an aberrant enhancement of the striatal direct

pathway, a feature, as we have seen, shared with drug addiction (Cenci, 2007; Jenner, 2008; Murer and Moratalla, 2011). The intriguing similarities between LID and the responses to drugs of abuse are also evident for what ERK signaling is concerned. Indeed, this signaling pathway is massively upregulated in the striatum of dyskinetic animals. To our knowledge, the induction of phosphorylated ERK1/2 in D1 receptor expressing MSN is the strongest ever detected not only in brain but in the whole body, accounting to up 50-fold increase over basal levels (as a comparison, high dose of cocaine can lead to a 10-fold increase). This enormous activation of ERK signaling is a combination of the engagement of the larger share of D1R expressing MSN and also, to a lesser extent, of an increase of the signal in already activated cells (Gerfen et al., 2002; Pavon et al., 2006; Westin et al., 2007). The specificity of the cell-type implicated in ERK activation was initially demonstrated pharmacologically but then it was confirmed using the BAC transgenic mice mentioned above as well as by causing gene ablation of the D1 receptors (Santini et al., 2008, 2009; Darmopil et al., 2009). As expected from the initial observations, pharmacological inhibition of ERK using SL327 or a partial blockade of Ras activity using statins results in a significant attenuation of LID onset (Santini et al., 2007; Schuster et al., 2008). More recently, also the Ras-GRF1 mutant mouse which was previously shown to be involved in memory and in drug addiction has been used to test the role of this molecule in LID. Indeed, a strong reduction in the AIM profile of Ras-GRF1 KO animals was observed together with a very significant attenuation of ERK1/2 activity in the dorsal striatum of these animals (Fasano et al., 2010). Interestingly, that work also showed that a combined treatment with suboptimal doses of SL327 (10 mg/kg), a condition which *per se* is ineffective in wild-type animals, causes an almost complete reduction of the dyskinetic symptoms.

All these data strongly support the notion that a specific targeting of neuronal components of the Ras–ERK pathway, like Ras-GRF1, may lead to effective treatments of both drug addiction and LID. In that respect, the scientific community still lacks pharmacological tools which go beyond SL327 and can target other molecular components besides MEK1/2. An interesting option is the development of cell permeable peptides that readily cross the brain–blood barrier and cause reversible disruptions of protein–protein interactions (Patel et al., 2007; Heitz et al., 2009). For instance, one can imagine that a Ras-GRF1 specific cell permeable peptide would be a valuable tool to study the role of this molecule in diverse behavioral processes, from memory formation, reconsolidation, and extinction, as well as for the development of treatments for the above mentioned diseases. At the same time, it is imperative to further advance in the development of cell-specific genetic tools to target crucial components of the Ras–ERK pathway with a tight temporal and spatial control. One step in that direction is the availability of BAC transgenics expressing CRE recombinase in a cell and brain area specific manner. For instance, the recent report in which DARPP-32, a signal integrator which in the striatum also controls ERK signaling, was either specifically targeted in the MSN of the direct or the indirect pathway lead to changes in the responses to cocaine/L-DOPA or to haloperidol, respectively (Bateup et al., 2010). Also, a very significant leap forward would be represented by the combination of conditionally targeted mouse mutants with cell-specific CRE delivery via viral vectors which would also allow us to achieve an excellent temporal control of gene expression. At present though, since some limitations still apply to validate viral vectors with *bona fide* cell-specific promoters, probably the best option would be to use BAC transgenic lines expressing CRE in combination with vectors conditionally expressing either dominant negative constructs, cell permeable peptides, or small interfering RNAs (shRNA), as recently suggested (Papale et al., 2009). These multiple approaches should be able to address the major remaining question linking drug addiction, LID and the Ras–ERK pathway in the striatum: why is only the direct pathway affected in these diseases? The specificity certainly lies on the hyperactivation of the D1 receptors in response to L-DOPA or to drugs like cocaine but this type of reasoning is rather circular and provides little explanation. So far, there is no evidence that any component of the Ras–ERK pathway is differentially expressed in the two striatal cell populations. Thus, it is difficult to judge whether the activation of Ras–ERK in D1R expressing cells is just a "minor" consequence of upstream events or plays a more "instructive" role. Certainly, gene ablation of D1R completely block ERK activation and downstream events in the striatum and affects not only LID but also learning and LTP in the hippocampus, as well as cocaine self-administration (Caine et al., 2007; Darmopil et al., 2009; Ortiz et al., 2010). An interesting experiment in the direction to solve this problem would be to force over-expression of one of Ras–ERK elements, e.g., Ras-GRF1 (which is normally in both MSN types) in the indirect pathway, which is silent in LID and in response to cocaine, and see whether that is sufficient to readjust the system. In addition, it would be important to knockdown Ras-GRF1 individually in each compartment and to express constitutively active mutations in the pathway (e.g., Ras G12V) and to verify the effect at the behavioral level. Both loss and gain of function experiments will be crucial but certainly demanding from the technological point of view. However, in our opinion, they will represent a necessary new level of investigation to tackle the complexity of Ras–ERK signaling in behavior which will keep us busy for at least additional 15 years.

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Mice lacking Ras-GRF1 show contextual fear conditioning but not spatial memory impairments: convergent evidence from two independently generated mouse mutant lines

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Ras-GRF1 is a neuronal specific guanine exchange factor that, once activated by both ionotropic and metabotropic neurotransmitter receptors, can stimulate Ras proteins, leading to long-term phosphorylation of downstream signaling. The two available reports on the behavior of two independently generated Ras-GRF1 deficient mouse lines provide contrasting evidence on the role of Ras-GRF1 in spatial memory and contextual fear conditioning. These discrepancies may be due to the distinct alterations introduced in the mouse genome by gene targeting in the two lines that could differentially affect expression of nearby genes located in the imprinted region containing the *Ras-grf1* locus. In order to determine the real contribution of Ras-GRF1 to spatial memory we compared in Morris Water Maze learning Brambilla's mice with a third mouse line (GENA53) in which a non-sense mutation was introduced in the Ras-GRF1 coding region without additional changes in the genome and we found that memory in this task is normal. Also, we measured both contextual and cued fear conditioning, which were previously reported to be affected in Brambilla's mice, and we confirmed that contextual learning but not cued conditioning is impaired in both mouse lines. In addition, we also tested both lines for the first time in conditioned place aversion in the Intellicage, an ecological and remotely controlled behavioral test, and we observed normal learning. Finally, based on previous reports of other mutant lines suggesting that Ras-GRF1 may control body weight, we also measured this non-cognitive phenotype and we confirmed that both Ras-GRF1 deficient mutants are smaller than their control littermates. In conclusion, we demonstrate that Ras-GRF1 has no unique role in spatial memory while its function in contextual fear conditioning is likely to be due not only to its involvement in amygdala functions but possibly to some distinct hippocampal connections specific to contextual learning.

Keywords: Ras-GRF1, Ras-ERK, spatial memory, fear conditioning, intellicage, body weight reduction

Memory is a high level brain function that allows organisms to modify their behavior in a way that is adaptive to the environment, increasing hence their probability to survive. It can be categorized in many subtypes and in different processes, but its main subdivision is the one in short-term memory (STM) and long-term memory (LTM), based on the duration of memory retention (Squire et al., 1993; Milner et al., 1998; Squire, 2004; D'isa et al., 2011). The first one, STM, which has a duration ranging from minutes to hours, does not require protein synthesis and is mainly based on the regulation of already existing proteins. However, in order to form a LTM, which can last days, months, years, or even decades, gene expression and *de novo* protein synthesis are required (Davis and Squire, 1984; McGaugh, 2000).

One of the major molecular cascades implicated in LTM formation is the Ras-controlled mitogen-activated protein kinase (MAPK)–extracellular regulated kinase (ERK) pathway (Blum and Dash, 2009). The Ras-ERK pathway was originally described as regulating cell proliferation and differentiation (Boulton et al., 1991; Marshall, 1995; Malumbres and Barbacid, 2003; Fernandez-Medarde and Santos, 2011a), but in the last 15 years its role in synaptic plasticity and memory consolidation has become evident. The canonical cascade is either brought about by neurotransmitters and their ionotropic and metabotropic receptors or by growth factors that act on receptor tyrosine kinases. This leads to the sequential activation of the Ras subfamily of small GTPases, Raf (the MAP kinase kinase kinase, MAPKKK), MEK (MAPKK) and finally ERK1 and ERK2, the two main MAPKs. ERK1/2 then translocate into the nucleus where they cause either directly or indirectly the activation of transcription factors like Elk-1, c-Myc, and CREB.

ERK1/2 activation is an essential requirement for synaptic plasticity (Orban et al., 1999; Mazzucchelli and Brambilla, 2000; Adams and Sweatt, 2002; Thomas and Huganir, 2004; Davis and Laroche, 2006; Peng et al., 2010; Fasano and Brambilla, 2011). The first evidence that this MAPK signaling is necessary for long-term potentiation (LTP) came in late 1990s from the work of English and Sweatt on hippocampal area CA1 (English and Sweatt, 1996, 1997), but since then similar findings have been made in many brain areas, including dentate gyrus (Coogan et al., 1999), amygdala (Huang et al., 2000), and visual cortex (Di Cristo et al., 2001).

Likewise, MAPK–ERK signaling is a fundamental requisite for LTM formation in the hippocampus. Pharmacological and genetic manipulations have demonstrated its role in several different types of memory. Administration of MEK inhibitor PD98059, for example, in entorhinal cortex impaired inhibitory avoidance (Walz et al., 1999) and spatial memory in Morris water maze (MWM; Hebert and Dash, 2002), while injected intracerebroventricularly it caused a deficit in conditioned taste aversion (Swank, 2000) and object recognition memory (Kelly et al., 2003). Systemic administration of SL327 (a MEK inhibitor which is able to cross the blood–brain barrier) led to impairments in MWM spatial memory and fear memory (contextual fear conditioning was completely blocked, while cued fear conditioning was attenuated; Atkins et al., 1998; Selcher et al., 1999). Some genetic manipulations confirm these results, such as the MEK1 KO mice which have reduced fear memory (Shalin et al., 2004) or mice with a homozygous null mutation for RIN1, a negative regulator of Ras, that have an enhancement in fear conditioning (Dhaka et al., 2003). In other cases, genetic ablation of components of the Ras-ERK pathway have led to more complex scenarios, see for instance the ERK1 KO mice or the NF1 mutants (Silva et al., 1997; Costa et al., 2002; Mazzucchelli et al., 2002; Cui et al., 2008; Fasano and Brambilla, 2011).

In the present work, we used mutant mice lacking the Ras guanine nucleotide-releasing factor 1 (Ras-GRF1; Fernandez-Medarde and Santos, 2011b). Ras is a small G-protein that is inactive until it is bound to GDP (Ras-GDP) and becomes active when it binds to GTP (Ras-GTP), acting as a molecular switch for the MAPK–ERK pathway (Boguski and McCormick, 1993). The shift from the inactive to the active state is mediated by guanine nucleotide exchange factors (GEFs), while the reverse change is promoted by GTPase-activating proteins (GAPs). Ras-GRF1 is a neuronal specific Ras-GEF, exclusively expressed in post-natal, fully differentiated neurons. The behavioral phenotype of Ras-GRF1 deficient mice (MGI nomenclature: Ras-grf1^{tm1Kln}) was first described in a paper by Brambilla et al. (1997). Mutant mice showed a clear dissociation between amygdala-dependent memory (fear conditioning and inhibitory avoidance), which was impaired, and hippocampus-dependent spatial memory in MWM, which resulted normal. Electrophysiology confirmed that LTP was compromised in basolateral amygdala (BLA) but not in CA1 region of hippocampus. Few years later, a study by Giese et al. (2001) found different results testing another knock-out mouse line for Ras-GRF1 (MGI nomenclature: Ras-grf1^{tm1Sva}). Amygdala-dependent memory (inhibitory avoidance and contextual fear conditioning) was normal, while a memory deficit was present in MWM. These mutants were also impaired in hippocampus-dependent contextual discrimination and the social transmission of food preference tasks.

To clarify this controversy, we used a third mutant line, GENA53 (MGI nomenclature: Ras-grf1^{enu1H}), which are ENU mutated mice with a non-sense point mutation that makes the Ras-GRF1 gene inactive with the minimum disturbance to the genome (Clapcott et al., 2003). The aim of this study is to conclusively define the role of Ras-GRF1 in learning and memory by simultaneously testing the two independent mutant lines Ras-GRF1 KO and GENA53 for spatial memory in MWM, contextual and cued fear conditioning, and conditioned corner avoidance in the recently developed system Intellicage.

MATERIALS AND METHODS

ANIMAL CARE

Male and female virgin mice were housed in separate sex and mixed genotype groups of 2–5 each in standard transparent polypropylene cages for mice (L × W × H: 33 cm × 15 cm × 13 cm) endowed with a sawdust bedding. Commercial pellet mice food and bottled tap water were available *ad libitum*. Animals were kept in a fixed light/dark cycle of 12 h:12 h, with lights on at 9:00 AM. Environment was temperature-controlled (21.5 ± 1°C) and humidity-controlled (40 ± 10%). Cages were inspected twice a week and changed weekly, but only before a day without behavioral testing. Adult (3–5 months) mice were used for the experiments. For the MWM both males and females, in the 50% proportion for the four genotypes, were used. Subsequent statistical analysis confirmed that no sex differences existed. In Intellicage, only females were used because less aggressive. Finally, in fear conditioning only males were used. All the behavioral tests were performed in a quiet and dimly lit room, different from the housing room. Animal care and experiments were conducted in accordance to the ethical guidelines expressed in the European Union directives, approved by the local Institutional Animal Care and Use Committee (IACUC) of the Istituto Scientifico San Raffaele and communicated to the national Ministry of Health as required by the Italian law and by the relevant European regulations.

MORRIS WATER MAZE

The MWM test was performed in a circular pool of 1.5 m in diameter, which was filled with water at the temperature of 26 ± 2°C. The water was made opaque by the addition of 2 L of full-cream long-life (UHT) milk, in order to render the submerged platform invisible to the mice. Extra-maze spatial cues were fixed on the walls surrounding the pool. During the training phase a transparent Plexiglas square platform (12 cm × 12 cm) was hidden in the target quadrant (TQ) 1 cm below the level of the water (the choice of the TQ was randomized across mice). In each trial animals were placed in the pool at specific release points (which were randomized across trials and mice) and were let to swim until they found the hidden platform or they reached 120 s of time. Escape latencies and swim tracks were recorded with the videotracking software SMART version 2.0 (Panlab, Barcelona, Spain)[1]. The spatial training lasted 24 trials (4 days with 6 trials per day). On the

[1] www.panlab.com

fifth day the test phase for memory consolidation was carried out. The hidden platform was removed and mice were tested in a single probe trial, which lasted 60 s and during which the swim tracks and the times spent in the TQ, zone, and platform were recorded with SMART. On the sixth day memories were reactivated in a 60-s session, during which the animals could freely swim in the pool without platform. Finally, 24 h later, on the seventh day, in order to evaluate memory reconsolidation mice were re-tested in a new probe trial identical to the one performed on day 5.

INTELLICAGE

Apparatus and software

The Intellicage apparatus (NewBehavior AG, Zurich, Switzerland)[2] is placed in a polycarbonate type III cage (20.5 cm high, 58 cm × 40 cm top, 55 cm × 37.5 cm bottom, Tecniplast, 2000P, Buguggiate, Italy) and accommodated up to 16 mice. It is covered by an aluminum top with a food rack. Its floor is covered with bedding and four central triangular red shelters (Tecniplast, Buguggiate, Italy) are provided. Four triangular conditioning chambers (15 cm × 15 cm × 21 cm) are fitted in the four cage corners and can be accessed by one mouse at a time. Each chamber contains two drinking bottles, accessible via two round openings (13 mm diameter) with motorized doors. Three multi-color LEDs are mounted above each door and the ceiling of each chamber contains a motorized valve that can deliver air puffs. Mice that access a chamber are identified by a circular RIFD antenna at its entrance (30 mm inner diameter) and the duration of their visit is monitored by a temperature sensor. During a visit, number and duration of individual nosepokes at each door are recorded using IR-beam sensors. Licking episodes at each bottle are monitored using lickometers (duration of the episode, number of licks, total contact time). Intellicages have an individual controller and are connected to a central PC running the software that permits to design and run experiments, as well as to analyze the recorded data (Designer, Controller, and Analyzer version 2.2.2, NewBehavior AG).

General procedures

RFID transponders (Datamars SA, Bedano, Switzerland) were injected subcutaneously in the dorso-cervical region under Isoflurane inhalation anesthesia. This was followed by 1 week recovery in groups of 10–12 mice in standard Type III cages (Tecniplast, Buguggiate, Italy) with food *ad libitum*. During the first week in Intellicage all doors were open providing free access to all eight drinking bottles (free adaptation). During the second week, all doors were closed but could be opened with a nosepoke for 5 s once per visit (nosepoke adaptation). This was followed by the corner avoidance task. During all phases of the experiment, mice were fed *ad libitum* with standard mouse food (Kliba Nafag 3430; Provimi Kliba AG, Kaiseraugst, Switzerland) and kept on aspen bedding (5 mm × 5 mm × 1 mm, Tapvei OY, Kortteinen, Finland) under controlled environmental conditions (temperature 21 ± 1°C, humidity 50 ± 5%, ambient lights off between 8:00 and 20:00).

Corner avoidance task

This task consisted of a training trial followed by two probe trials (test and re-test). All trials lasted 24 h and were separated from the previous trial by a 24-h retention interval outside Intellicage in a regular type III cage. During the last 18 h of the retention interval water was removed. During the training trial, each mouse was assigned a target corner (avoiding the most and least visited corner during pre-training baseline) in which nosepokes triggered a 1-s air puff instead of opening the door. During probe trials, no air puffs were given and doors opened in response to nosepokes in all corners, as during nosepoke adaptation. After the second probe trial, the mice were left in the Intellicage without air puffs for further 3 days. To assess extinction of the learned response, avoidance of the target corner was again evaluated during the last 24 h of this period. Avoidance of the target corner was quantified as 100% × nosepokes to target corner/total nosepokes. The distribution of poke-less visits was also evaluated but was not affected by training. Within a visit to a corner only the first nosepoke was counted since multiple nosepokes during the same visit are not related to memory.

FEAR CONDITIONING

Apparatus and procedures

Four mice were tested in parallel in an Actimetrics FreezeFrame video-based Conditioned Fear System[3]. The conditioning chambers (175 mm deep × 180 mm wide × 280 mm high) are enclosed in ventilated and sound-attenuated cabinets and have a floor consisting of stainless steel rods permitting the application of current. The training session consisted of a 60-s pre-exposure to the training context immediately followed by three 60-s training trials consisting of a 2500-Hz 85 dB tone conditioned stimulus (CS) lasting 30 s, co-terminating with a 2-s 0.25 mA foot shock (US), and followed by a 300-s interval. To assess memory consolidation 24 h after training, the mice were re-exposed to the training chamber for 120 s without activation of the CS or US (context test). Thereafter the floor of the conditioning chamber was covered with plastic and some bedding material and a pebble were added. The mice were pre-exposed to this modified context for 60 s which was immediately followed by a 60-s CS presentation (tone test). To assess memory reconsolidation, both test sessions were repeated 6 days later (context and tone re-test).

Data analysis

Freezing (absence of movement aside from respiration) was quantified automatically by the FreezeFrame software subtracting subsequent images recorded by the ceiling-mounted IR video cameras in the conditioning chambers. Bouts of 1.0 s were used to define percentage freezing and movement thresholds were set at 20 (training and context test) or 8 units (tone test). The training effect was evaluated by comparison of percentage freezing during the last training cycle with freezing during the first CS presentation plus the last 30 s of pre-exposure. The US-context association was assessed by comparing percentage freezing during pre-exposure

[2]www.newbehavior.com

[3]www.actimetrics.com

with percentage freezing during the context test. The US–CS association (consolidation and reconsolidation) was evaluated by comparing percentage freezing during the pre-CS and CS phases of the tone test. Additional measures were: percentage freezing during context pre-exposure (unconditioned freezing to the training context), percentage freezing during the first tone presentation (unconditioned freezing to the tone), percentage freezing during the pre-CS phase of the tone test (generalized freezing to the new context).

RESULTS

MORRIS WATER MAZE LEARNING IS NORMAL IN BOTH Ras-GRF1 DEFICIENT MOUSE LINES

In order to assess spatial memory mice were tested in the (MWM; Morris et al., 1982), in which animals have to swim in a circular pool with opaque water and learn to find a submerged platform that is hidden under the water surface of one of the four quadrants of the pool, called the "TQ." Mice were trained for 24 trials (4 days with six trials per day). **Figure 1** illustrates the learning curves during the 4 days of training (data are shown as couples of trials). One-way ANOVA for repeated measures performed on data from each of the four genotype groups revealed that all the four groups learned, since there is a significant decrease in the escape latencies [Ras-GRF1 KO: $F(11,121) = 9.323$, $p < 0.0001$; Ras-GRF1 WT: $F(11,121) = 19.268$, $p < 0.0001$; GENA53: $F(11,121) = 17.799$, $p < 0.0001$; GENA53 WT: $F(11,121) = 9.030$, $p < 0.0001$]. Comparing the learning curves by means of a two-way ANOVA for repeated measures, Ras-GRF1 KO and GENA53 mutant mice resulted both undistinguishable from their respective WT littermates (Bonferroni's *post hoc*, Ras-GRF1 KO vs. Ras-GRF1 WT: $p = 1$; GENA53 vs. GENA53 WT: $p = 1$), demonstrating their learning was of the same entity and velocity. Moreover they were comparable between themselves (Ras-GRF1 KO vs. GENA53: $p = 0.621$), as were the wild-type controls (Ras-GRF1 WT vs. GENA53 WT: $p = 0.264$). There was instead a significant main effect of the training [Trial: $F(11,484) = 47.100$, $p < 0.0001$].

After the 4 days of training, on the fifth day the hidden platform was removed and the animals were challenged with the memory test, which consisted in a single probe trial. **Figure 2A** shows the percentages of time spent swimming in the TQ. Learning is considered significant if the percentage of time for the TQ is significantly superior than the chance level (which is 25%, since the pool is divided into four equal quadrants) and if this percentage is significantly higher than the ones for the remaining three control quadrants (opposite quadrant, OQ; right quadrant, RQ; left quadrant, LQ). All four genotypes showed a significant preference for the TQ, demonstrating to have learned the task. For each genotype the percentage of time spent in the TQ was significantly higher than chance level [One-sample t-test, Ras-GRF1 WT: $t(11) = 5.652$, $p < 0.0001$; Ras-GRF1 KO: $t(11) = 5.838$, $p < 0.0001$; GENA53 WT: $t(11) = 6.555$, $p < 0.0001$; GENA53: $t(11) = 6.507$, $p < 0.0001$] and than the percentages for the other quadrants (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, TQ vs. OQ: all $p < 0.0001$; TQ vs. LQ: all $p < 0.009$; TQ vs. RQ: all $p < 0.013$). Moreover one-way ANOVA revealed that the percentages of time spent in the TQ did not differ between genotypes, indicating that the levels of learning of the mutants were analogous to the ones of the wild-type littermates [$F(3,44) = 0.487$, $p = 0.693$].

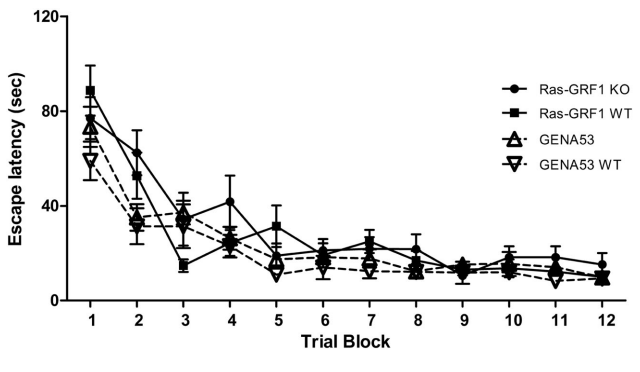

FIGURE 1 | Spatial learning in the Morris water maze. Escape latencies (mean ± SEM) during the 4 days of training are illustrated (results are shown by couples of trials). Ras-GRF1 WT ($n = 12$); Ras-GRF1 KO ($n = 12$); GENA53 WT ($n = 12$); GENA53 ($n = 12$).

These data prove that Ras-GRF1 KO and GENA53 have no impairment in spatial memory consolidation. As consolidation and reconsolidation often dissociate in their molecular bases (Alberini, 2005), we also wanted to test these mutant mice for spatial memory reconsolidation. On the sixth day, animals underwent memory reactivation and 24 h later, on the seventh day, they were exposed to a new probe trial. All the mice still maintained memory of the location of the hidden platform (**Figure 2B**), as times spent in TQ were again above chance level [One-sample t-test, Ras-GRF1 WT: $t(11) = 3.828$, $p = 0.003$; Ras-GRF1 KO: $t(11) = 3.269$, $p = 0.007$; GENA53 WT: $t(11) = 6.500$, $p < 0.0001$; GENA53: $t(11) = 4.122$, $p = 0.002$] and superior than the times spent in the opposite quadrant (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, Ras-GRF1 WT: $p = 0.005$; Ras-GRF1 KO: $p = 0.011$; GENA53 WT: $p < 0.0001$; GENA53: $p = 0.002$). One-way ANOVA confirmed that Ras-GRF1 KO and GENA53 did not have their memory disrupted after reactivation and had intact reconsolidation processes, since the times spent in the TQ by the four genotypes resulted equivalent [$F(3,44) = 1.294$, $p = 0.288$].

As Ras-GRF1 KO and GENA53 mutants, although lacking Ras-GRF1, resulted normal in memory consolidation and reconsolidation considering the TQ, we decided to analyze the performance of these animals with a more demanding requirement, learning within the target zone (TZ), which represents only 12.5% of the total area of the pool (half a quadrant), in order to detect even slight memory impairments. On the fifth day, in the probe trial for consolidation, mice of all four genotypes revealed a significant preference for the TZ (**Figure 2C**). Percentages of time spent in it were significantly superior than chance level [One-sample t-test, Ras-GRF1 WT: $t(11) = 6.526$, $p < 0.0001$; Ras-GRF1 KO: $t(11) = 5.491$, $p < 0.0001$; GENA53 WT: $t(11) = 7.818$, $p < 0.0001$; GENA53: $t(11) = 5.805$, $p < 0.0001$] and than the percentages of time spent in the other zones (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, TZ vs. OZ: all $p < 0.001$; TZ vs. LZ: all $p < 0.014$; TZ vs. RZ: all $p < 0.018$). In addition the comparison of the animals' performances by means of one-way ANOVA indicated that the levels of learning of Ras-GRF1 KO

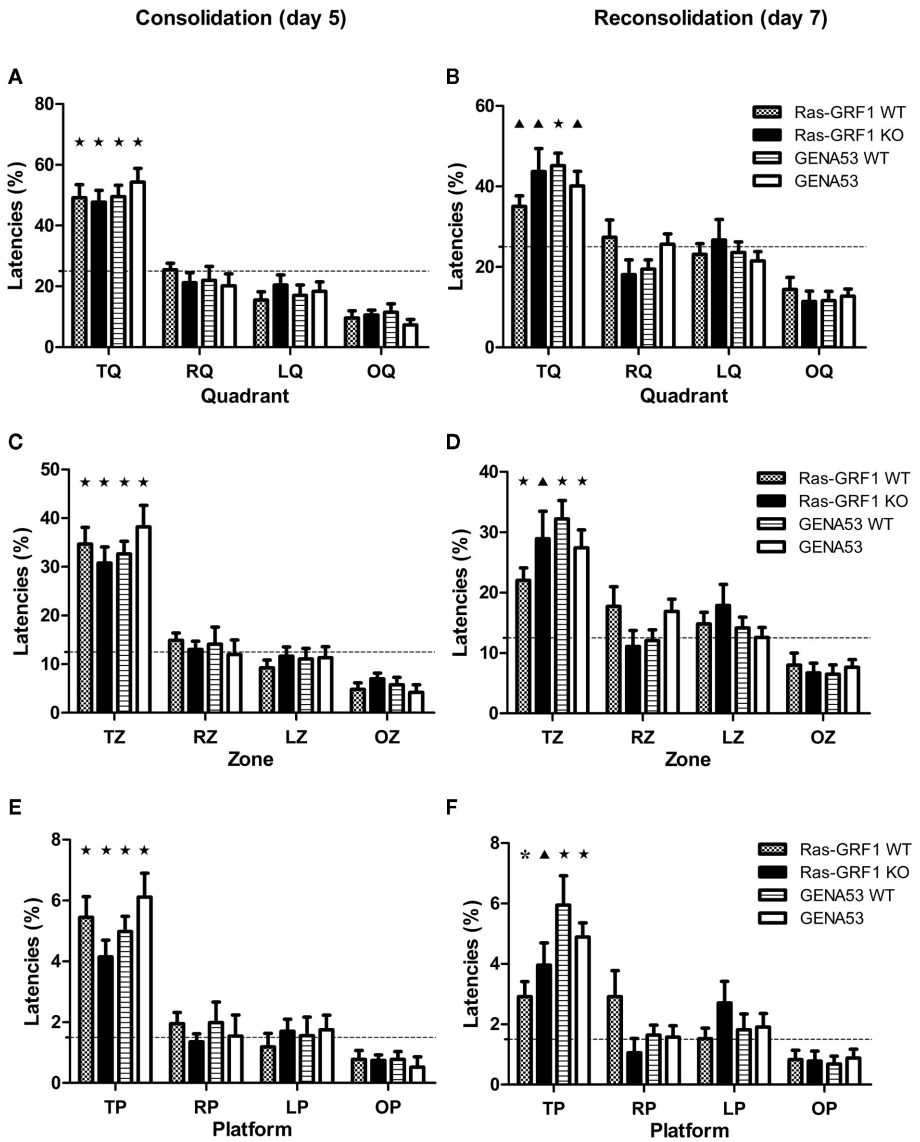

FIGURE 2 | Spatial memory in the Morris water maze. Percentages (mean ± SEM) of time spent in target quadrant (first row: **A,B**), target zone (second row: **C,D**), and target platform (third row: **E,F**). Spatial memory consolidation (day 5 probe trial) is shown in the left column **(A,C,E)**, while spatial memory reconsolidation (day 7 probe trial) is shown in the right column **(B,D,F)**. Ras-GRF1 WT ($n = 12$); Ras-GRF1 KO ($n = 12$); GENA53 WT ($n = 12$); GENA53 ($n = 12$). Percentages in target area are compared against chance level. Dashed lines represent chance level for each area (quadrant: 25%; zone: 12.5%; platform: 1.5%). T, target; R, right; L, left; O, opposite. *P*-value symbols: black star = $p < 0.001$; black triangle = $p < 0.01$; asterisk = $p < 0.05$.

and GENA53 were equal to the ones of their wild-type littermates [$F(3,44) = 0.833$, $p = 0.483$].

On the seventh day, in the probe trial for reconsolidation, none of the four genotypes showed to have its memory disrupted by the reactivation of the sixth day (**Figure 2D**). Times spent in TZ were still higher than chance level [One-sample *t*-test, Ras-GRF1 WT: $t(11) = 4.674$, $p = 0.001$; Ras-GRF KO: $t(11) = 3.607$, $p = 0.004$; GENA53 WT: $t(11) = 6.410$, $p < 0.0001$; GENA53: $t(11) = 4.975$, $p < 0.0001$] and than the times spent in the opposite zone (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, Ras-GRF1 WT: $p = 0.006$; Ras-GRF1 KO: $p = 0.018$; GENA53 WT: $p < 0.0001$; GENA53: $p = 0.002$). Levels of memory were equivalent across genotypes [One-way ANOVA, $F(3,44) = 1.654$, $p = 0.191$].

Finally, since also in the TZ test GENA53 and Ras-GRF1 KO mice demonstrated to be able to learn, we decided to challenge the animals with a most stringent analysis, the target platform test, in which a significantly smaller surface is considered, merely the area that would have been occupied by the platform that has been removed for the probe trial, which represents only 1.5% of the pool, hence a threshold that is over 16 times more stringent than the TQ and over eight times more than the TZ. We found that the mutant mice passed also this test (**Figure 2E**). All genotypes spent in the target platform a

percentage of time that was higher than chance level [One-sample t-test, Ras-GRF1 WT: $t(11) = 5.779$, $p < 0.0001$; Ras-GRF1 KO: $t(11) = 4.777$, $p = 0.001$; GENA53 WT: $t(11) = 7.099$, $p < 0.0001$; GENA53: $t(11) = 5.867$, $p < 0.0001$] and than the one spent in the opposite platform (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, Ras-GRF1 WT: $p = 0.002$; Ras-GRF1 KO: $p = 0.001$; GENA53 WT: $p < 0.0001$; GENA53: $p < 0.0001$). Performances of Ras-GRF1 KO and GENA53 mice did not differ from the ones of their wild-type littermates even in this stringent test [One-way ANOVA, $F(3,44) = 1.669$, $p = 0.187$].

In the probe trial for reconsolidation the results were the same as for consolidation (**Figure 2F**). All the animals passed the test, given that the time spent in the target platform was superior than chance level [One-sample t-test, Ras-GRF1 WT: $t(11) = 2.847$, $p = 0.016$; Ras-GRF1 KO: $t(11) = 3.390$, $p = 0.006$; GENA53 WT: $t(11) = 4.582$, $p = 0.001$; GENA53: $t(11) = 7.241$, $p < 0.0001$] and than the one spent in the opposite platform (One-way ANOVA for repeated measures, Bonferroni's *post hoc*, Ras-GRF1 WT: $p = 0.034$; Ras-GRF1 KO: $p = 0.030$; GENA53 WT: $p = 0.003$; GENA53: $p < 0.0001$). Again, performances of the mutants were comparable to the ones of the wild-types and between them (One-way ANOVA, Bonferroni's *post hoc*, Ras-GRF1 KO vs. Ras-GRF1 WT: $p = 1$; GENA53 vs. GENA53 WT: $p = 1$; Ras-GRF1 KO vs. GENA53: $p = 1$).

NORMAL CONDITIONED PLACE AVOIDANCE LEARNING IN THE INTELLICAGE OF THE TWO RAS-GRF1 MUTANT LINES

Mice were tested in Intellicage for conditioned place avoidance. Intellicage is a recently developed system (Galsworthy et al., 2005; Lipp, 2005; see text footnote 2) that allows automated measurement of mice's spontaneous behavior and cognitive abilities (Knapska et al., 2006; Kiryk et al., 2008; Jaholkowski et al., 2009; Viosca et al., 2009; Barlind et al., 2010; Voikar et al., 2010). With this system animals are tested while living in their cage, without being stressed by the manipulation of human experimenters and without altering their normal social life. Each Intellicage contains fours identical corners, which can be accessed by only one mouse at a time. The identity of mice entering a corner is revealed by passive transponders implanted subcutaneously.

In the conditioned place avoidance each corner contains two water bottles located behind a door that can be opened by a nosepoke. After an adaptation phase in which the animals learn that the doors can be opened by nosepokes (nosepoke adaptation), for each mouse a corner is selected randomly for avoidance conditioning. In this corner the nosepoke, instead of opening the door to allow drinking, triggers an air puff, a non-painful punishment. After 24 h of conditioning mice are removed from Intellicages. The day after the animals, water-deprived for 18 h, undergo the test session for their 24 h-memory, which lasts other 24 h. In this session in all corners the doors can be opened by a nosepoke and there are no punishments. Mice that have learned the task avoid the corner associated with the air puffs and drink from the other three. Percentages of visits with nosepokes (or first nosepokes; see Materials and Methods) to the trained corner are recorded as an index of learning (visits without nosepokes are not influenced by training).

Learning did not appear to be affected by the mutations, as all genotypes developed a strong avoidance for the trained corner (**Figure 3**). Percentage of nosepokes in the conditioned corner during the test phase was significantly lower than the one during the nosepoke adaptation phase [T-test for paired-samples, Ras-GRF1 WT: $t(11) = 5.271$, $p = 0.0003$; Ras-GRF1 KO: $t(11) = 7.710$, $p < 0.0001$; GENA53 WT: $t(11) = 4.809$, $p = 0.0006$; GENA53: $t(10) = 6.186$, $p = 0.0001$], as it was already the one of the training phase [T-test for paired-samples, Ras-GRF1 WT: $t(11) = 6.090$, $p < 0.0001$; Ras-GRF1 KO: $t(11) = 9.333$, $p < 0.0001$; GENA53 WT: $t(11) = 5.056$, $p = 0.0004$; GENA53: $t(10) = 8.464$, $p < 0.0001$]. Furthermore, the percentages of nosepokes were comparable between the mutant animals and their wild-type control littermates in the training phase (One-way ANOVA, Bonferroni's *post hoc*, Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.184$; GENA53 WT vs. GENA53: $p = 1$) and in the test phase (One-way ANOVA, Bonferroni's *post hoc*, Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.596$; GENA53 WT vs. GENA53: $p = 1$).

After the memory consolidation test the mice were removed again from Intellicages, placed in standard cages for 24 h (water-deprived in the last 18 h) and then returned to Intellicages for a new 24-h probe trial, this time to test memory reconsolidation. Subsequently, animals were kept in Intellicages for other 3 days to assess extinction.

In the second probe trial the mutated mice were again comparable to their wild-type littermates, revealing that Ras-GRF1 KO and GENA53 mutants do not have impairments in reconsolidation (One-way ANOVA, Bonferroni's *post hoc*, Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 1$; GENA53 WT vs. GENA53: $p = 1$). For all four genotypes learning was still significant, compared with the baseline level of nosepokes recorded during the adaptation phase [T-test for paired-samples, Ras-GRF1 WT: $t(11) = 10.632$, $p < 0.0001$; Ras-GRF1 KO: $t(11) = 8.263$, $p < 0.0001$; GENA53 WT: $t(11) = 3.969$, $p = 0.002$; GENA53: $t(10) = 8.148$, $p < 0.0001$]. Finally, after the 3-day extinction protocol, in which the air puff punishment had been removed, we

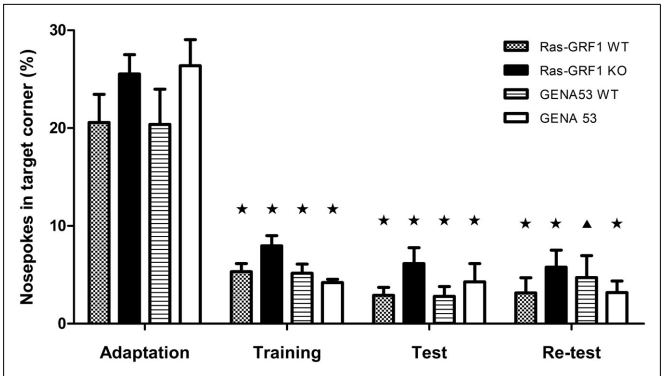

FIGURE 3 | Conditioned corner avoidance in Intellicage. Percentages (mean ± SEM) of first nosepokes (i.e., visits with a nosepoke) in the trained corner during the adaptation, training, test, and re-test phases. Ras-GRF1 WT ($n = 12$); Ras-GRF1 KO ($n = 12$); GENA53 WT ($n = 12$); GENA53 ($n = 11$). Percentages of first nosepokes are compared with the basal percentages observed during the adaptation phase. *P*-value symbols: black star = $p < 0.001$; black triangle = $p < 0.01$; asterisk = $p < 0.05$.

found the mutants' percentages of nosepokes in the trained corner did not differ from the ones of their littermates, demonstrating that Ras-GRF1 KO and GENA53 mice are not impaired in extinction learning (One-way ANOVA, Bonferroni's *post hoc*, Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.740$; GENA53 WT vs. GENA53: $p = 1$).

IMPAIRED LEARNING OF FEAR CONDITIONING IN THE TWO MUTANT LINES

Fear conditioning is a procedure based on Pavlovian associative learning, widely used to study emotional memory (Fendt and Fanselow, 1999; LeDoux, 2000; Maren, 2001; Fanselow and Poulos, 2005). Mice are exposed to an electric shock and learn to associate it with a neutral stimulus like the chamber in which they received the shock (contextual fear conditioning) or an auditory tone (cued fear conditioning). Both contextual and cued fear conditioning require the BLA whereas only contextual fear conditioning is hippocampus-dependent (Phillips and LeDoux, 1992). After having trained the animals we tested them for LTM and memory reconsolidation in both contextual and cued fear conditioning.

In the training phase both Ras-GRF1 KO and GENA53 mutants showed a deficit in learning (**Figure 4A**). Comparing the conditioned fear responses (measured as percentage of freezing) during the first presentation of the tone used as CS and the fear responses during the last CS, two-way ANOVA for repeated measures revealed a significant effect of training [$F(1,40) = 38.979$, $p < 0.0001$], a significant interaction training × genotype [$F(3,40) = 2.997$, $p = 0.042$], and a significant effect of genotype [$F(3,40) = 3.388$, $p = 0.027$]. LSD *post hoc* made clear that Ras-GRF1 KO mutants have a memory deficit (Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.028$), as GENA53 mutants have (GENA53 WT vs. GENA53: $p = 0.034$). Moreover, one-way ANOVA performed on the rates of learning (freezing to last CS – first CS) indicated a significant genotype effect [$F(3,40) = 3.201$, $p = 0.033$] and LSD *post hoc* analysis showed that both mutants have a memory impairment (Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.035$; GENA53 WT vs. GENA53: $p = 0.048$).

Twenty-four hours after the training mice were tested for contextual and cued fear memory consolidation (**Figure 4B**). In contextual fear conditioning we found a significant effect of context [Two-way ANOVA for repeated measures: $F(1,40) = 81.181$, $p < 0.001$], a significant context × genotype interaction [$F(3,40) = 3.112$, $p = 0.037$], and a significant genotype effect [$F(3,40) = 3.039$, $p = 0.040$]. For both mutants consistently, LSD *post hoc* displayed only a trend toward significance when they were compared with their respective wild-types (Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.075$; GENA53 WT vs. GENA53: $p = 0.068$). Nevertheless, when freezing responses of the mice were categorized on the basis of the performance (<5%: non-learners; 5–19,99%: bad learners; >20%: good learners), we discovered that the Ras-GRF1 KO and GENA53 mutants had both a strikingly greater proportion of non-learners (Ras-GRF1 KO: 45.5%; Ras-GRF1 WT: 0%; GENA53: 45.5%; GENA53 WT: 8.3%) and a much lower proportion of good learners (Ras-GRF1 KO: 45.5%; Ras-GRF1 WT: 80%; GENA53: 36.4%; GENA53 WT: 75%). Furthermore, comparison of the mean ratings of the performance (non-learners: 0; bad learners: 1; good learners: 2) by means of one-way ANOVA revealed a significant effect of genotype [$F(3,40) = 3.554$, $p = 0.023$]. LSD *post hoc* clarified that Ras-GRF1 KO and GENA53 mutants had both a significantly lower learning than their wild-type littermates (Ras-GRF1 WT vs. Ras-GRF1 KO: $p = 0.026$; GENA53 WT vs. GENA53: $p = 0.028$).

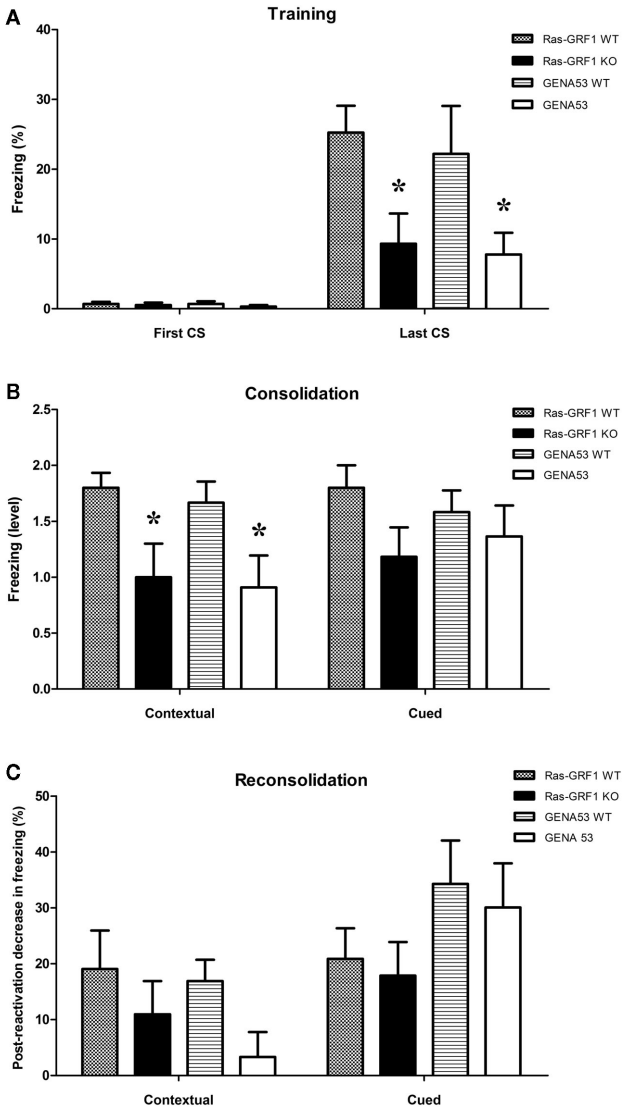

FIGURE 4 | Contextual and cued fear conditioning. Fear memory (means ± SEM) in the training **(A)**, test **(B)**, and re-test **(C)** phases. **(A)** Percentage of freezing at the beginning (first CS) and at the end (last CS) of the training. **(B)** Freezing level in the memory consolidation test (categorized by performance: 0 = non-learner; 1 = bad learner; 2 = good learner). **(C)** Decrease of freezing percentage in the memory reconsolidation test (percentage of freezing before reactivation minus percentage of freezing post-reactivation). Ras-GRF1 WT ($n = 10$); Ras-GRF1 KO ($n = 11$); GENA53 WT ($n = 12$); GENA53 ($n = 11$). Each mutant is compared with its respective wild-type by means of a *post hoc* comparison. *P*-value symbols: black star = $p < 0.001$; black triangle = $p < 0.01$; asterisk = $p < 0.05$.

In cued fear conditioning instead Ras-GRF1 KO and GENA53 mice did not show any memory consolidation impairment. Effect of tone presentation was significant, but the effect of genotype was not [Two-way ANOVA for repeated measures, Tone effect:

$F(1,40) = 95.354$, $p < 0.0001$; Genotype effect: $F(3,40) = 0.810$, $p = 0.496$]. Mean ratings of performance were also comparable across genotypes [One-way ANOVA: $F(3,40) = 0.803$, $p = 0.499$].

Six days after the memory consolidation tests mice were re-tested to evaluate also memory reconsolidation (which was assessed by comparing the memory levels shown in the test after 24 h with the ones of the re-test). For what concerns contextual fear memory reconsolidation, two-way ANOVA for repeated measures proved a significant effect of time [$F(1,40) = 22.706$, $p < 0.001$], but the effect of genotype was no more significant [$F(3,40) = 2.846$, $p = 0.050$] and neither was the time × genotype interaction [$F(3,40) = 1.764$, $p = 0.170$].

Similar results were obtained in the test for tone fear memory reconsolidation [Two-way ANOVA for repeated measures, Time: $F(1,40) = 54.198$, $p < 0.001$; Genotype: $F(3,40) = 1.515$, $p = 0.225$; Time × Genotype interaction: $F(3,40) = 1.238$, $p = 0.309$; **Figure 4C**].

Ras-GRF1 CONTROLS BODY WEIGHT DEVELOPMENT

It has previously been reported that the lack of Ras-GRF1 can lead to reduced body growth and development (Itier et al., 1998; Clapcott et al., 2003). Thus, we decided to test our mutant mice also from this point of view and compare the effect of the two mutations on a non-cognitive phenotype like body weight. However, in order to understand the involvement of our mutations in ponderal alterations, we measured the adult body weight of mutant mice from three genetic lines: the already mentioned Ras-GRF1 and GENA53 lines and a third additional line in which the mutants over-express Ras-GRF1, that is the Ras-GRF1, OE line (MGI nomenclature: Ras-grf1^{tm2Pds}; Yoon et al., 2005; Fasano et al., 2009; **Figure 5**).

For what concerns the Ras-GRF1 line, wild-type mice (4-month-old males only) showed a mean weight of 28.1 ± 1.0 g, ranging from a minimum of 19.1 g to a maximum of 40.0 g. Ras-GRF1 KO mice instead had a mean weight of 24.1 ± 0.5 g (range: 18.7 – 29.9 g), revealing a reduction of approximately 14% [Independent-samples t-test, $t(46) = -3.508$, $p = 0.001$]. Similarly, in the GENA53 line the wild-type mice exhibited a mean weight of 29.0 ± 0.7 g (range: 21.7 – 37.4 g), while the littermate GENA53 mutants showed a mean weight of 25.6 ± 0.9 g (range: 21.0 – 37.2 g), demonstrating a reduction of roughly 12% [Independent-samples t-test, $t(46) = -3.031$, $p = 0.004$]. Finally, in the Ras-GRF1 OE line the wild-types had a mean weight of 28.0 ± 0.6 g (range: 22.8 – 33.6 g), whereas the Ras-GRF1 OE mutants developed a mean weight of 34.7 ± 0.7 g (range: 29.8 – 41.4 g). In this case, in the mutants we observed an opposite effect on body weight: the range was shifted (both minimum and maximum were higher) and the mean weight was increased by approximately 24% [Independent-samples t-test, $t(46) = 7.181$, $p < 0.0001$].

Interestingly, the mean weight reductions observed in the two loss of function mutants, Ras-GRF1 KO and GENA53, were comparable among them [Independent-samples t-test, $t(46) = 0.422$, $p = 0.675$]. As a control, we also checked that the weights of the wild-type mice from the three different lines were all equivalent between them [One-way ANOVA: $F(2,71) = 0.466$, $p = 0.630$].

DISCUSSION

The present manuscript addresses a 10-year-old controversy originated by the contrasting results reported by Brambilla et al. (1997) and Giese et al. (2001). Brambilla et al. (1997) showed that spatial memory was intact in a mouse line deficient for Ras-GRF1 while both contextual and cued fear conditioning as well as fear-related forms of instrumental learning were found severely impaired. Consistently, Brambilla et al. (1997) found that two forms of hippocampal LTP were intact in these mouse mutants while LTP in the BLA was lost. On the contrary, Giese et al. (2001) reported that MWM learning was impaired in an independently generated deficient line for Ras-GRF1. In addition, Giese et al. (2001) also showed impaired learning in two additional hippocampal dependent tests, social transmission of food preference and contextual discrimination. These later findings suggested significant differences among the two mouse lines which were not strictly dependent on the loss of p140 Ras-GRF1 but rather ascribable

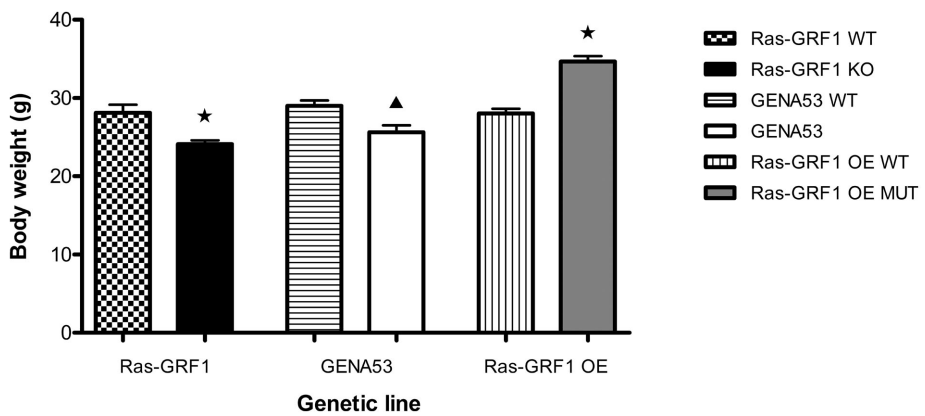

FIGURE 5 | Body weight. Total body weight (mean ± SEM) of adult mice from the three genetic lines Ras-GRF1, GENA53, and Ras-GRF1 OE. Ras-GRF1 WT ($n = 24$); Ras-GRF1 KO ($n = 24$); GENA53 WT ($n = 24$); GENA53 ($n = 24$); Ras-GRF1 OE WT ($n = 24$); Ras-GRF1 OE MUT ($n = 24$). Each mutant is compared with its respective wild-type by means of an independent-samples t-test. P-value symbols: black star = $p < 0.001$; black triangle = $p < 0.01$; asterisk = $p < 0.05$.

to genomic alterations, including the different position of the mutation caused within the *Ras-grf1* locus and/or the insertion of the neomycin resistance cassette. Indeed, the *Ras-grf1* gene is located within a genomic region which is maternally repressed through an imprinting mechanism and we and others demonstrated that the *Ras-grf1* gene is silenced in maternally derived alleles (Plass et al., 1996; Brambilla et al., 1997; Yoon et al., 2002). In order to conclusively define the role of Ras-GRF1 in various forms of LTM, we took advantage of a third mouse line, GENA53, in which Ras-GRF1 expression was abolished by chemical mutagenesis that inserted in the locus a single non-sense mutation, without introducing the neomycin resistance cassette which may potentially interfere with the expression of nearby genes (Clapcott et al., 2003). This line was preliminarily tested for MWM performance in 2009 and did not show deficits but it did manifest a loss of memory in a form of instrumental learning (passive avoidance), as for Brambilla's mice (Fasano et al., 2009). Therefore, in the present work we compared side by side MWM learning of both Brambilla's mice and the GENA53 line. We did not find any sign of impairment, neither in the acquisition, nor in the consolidation/reconsolidation processes, even considering very stringent learning criteria such as time swimming in the target zone area or in the target platform area. These data conclusively indicate that Ras-GRF1 dependent signaling is not *per se* essential for the formation of the memory trace in spatial learning and that the phenotype originally detected in the Giese's mice is likely due to the peculiar genomic location of the targeted mutation. However, since we did not tested Brambilla's and GENA53 mice in social transmission of food preference and in contextual discrimination, we cannot formally exclude that Ras-GRF1 may be implicated in these two tests which may depend on other components of the hippocampal circuitry, as we recently showed for novel object recognition and the perirhinal cortex (Berardi et al., 2011). Interestingly, at the cellular level, a more recent report on the Giese's mice indicated that hippocampal LTP was normal, while long-term depression (LTD) was found reduced (Li et al., 2006). These data were interpreted as a compensatory effect of Ras-GRF2, a close homolog, on hippocampal LTP, and our new data tend to confirm this interpretation also at the behavioral level. The role of the LTD effect found in the Giese's mice in behavior remains to be elucidated but clearly does not seem to be crucial for spatial learning.

The major emphasis of the original paper on Brambilla's mice was on the function of Ras-GRF1 in amygdala since both LTP in this structure and fear conditioning were found altered. A close comparison of Brambilla's and the GENA53 mice in the present manuscript essentially confirmed the phenotype associated with contextual fear but not with cued conditioning, suggesting interesting differences. Traditionally, both forms of fear conditioning have been linked to amygdala functions but the contextual version is also dependent on the hippocampus (Kim and Fanselow, 1992; Maren and Fanselow, 1996). Based on this interpretation we should suggest that hippocampal but not amygdalar functions are altered in both Brambilla's and the GENA53 mice. However, a possible alternative interpretation is that the protocol for cued conditioning used here is not sensitive enough to detect subtle differences. Indeed, here we used an intermediate protocol, with a three-tone/shock pairing, between the original Brambilla et al. (1997) report (five tone/shock pairings) and the Giese et al. (2001) one (one tone/shock pairing). Possibly, with a stronger protocol we would have been able to detect a phenotype in the cued version of the task as well. That would be more in line with the idea that MWM performance and hippocampal LTP are normal in most Ras-GRF1 mutant lines (Brambilla's and GENA53 are normal in MWM, Brambilla's and Giese's have normal LTP) and that MWM is also normal in a Ras-GRF1 over-expressing line, whose behavioral characterization has already been reported (Fasano et al., 2009). However, it is also important to consider the fact that the hippocampal circuitry and/or the hippocampal signaling mechanisms involved in contextual fear conditioning may be distinct from those used in spatial learning, leaving the possibility that other components of the hippocampal formation may be specifically impaired in fear learning in the Ras-GRF1 deficient mice, as previously suggested for other mutant lines (Mizuno and Giese, 2005) or based on lesion studies (Phillips and LeDoux, 1992, 1994, 1995).

Concerning this point, the fact that, at least in the Giese mice, LTD was found affected, already provides an interesting cellular phenotype which may underlie the contextual fear conditioning impairment.

The strongest behavioral phenotype detected in Brambilla's mice which was further confirmed in the GENA53 mice is the impairment of LTM in fear-related instrumental learning tasks such as passive and active avoidance (Brambilla et al., 1997; Fasano et al., 2009). Interestingly, an opposite phenotype (i.e., a memory gain) was found in the above mentioned Ras-GRF1 over-expressing mice, further supporting the notion that this signaling modulator of the Ras-ERK pathway is crucial for these types of learning. At present, although we cannot exclude a contribution of amygdala on the effect seen in instrumental learning, the available data suggest that the behavioral alterations of all Ras-GRF1 mutations are likely to be linked to an altered synaptic plasticity and abnormal cell signaling in the striatum, as we recently reported (Fasano et al., 2009). Thus, our observation that learning in the Intellicage is normal in both Brambilla's and the GENA53 mice is not entirely surprising. Certainly, conditioned place avoidance as assessed in this fully automated system is rather different than active and passive avoidance learning and indeed it may rely on different neural circuitry which may also include the hippocampus but not the dorsal striatum (Voikar et al., 2010).

One final comment is about the growth reduction originally observed in yet another Ras-GRF1 KO line (MGI nomenclature: Ras-grf1^{tm1Toc}) which was also confirmed in the original GENA53 report (Itier et al., 1998; Clapcott et al., 2003). Here, we show that also Brambilla's mice have a reduced body weight in the adult stage, as previously suggested but not formally reported (Orban et al., 1999). In addition, we have also included for the first time the body weight data of the Ras-GRF1 over-expressing mice (Fasano et al., 2009). The fact that the over-expressing mice weight more than controls is an independent confirmation that Ras-GRF1 is important for post-natal development of the body and that this may also be linked to its role in aging, as recently suggested (de Magalhaes, 2010; Borras et al., 2011).

Altogether, our data indicate that Ras-GRF1 is important for contextual fear-related memories but its involvement in spatial memory is likely to be masked by the presence of Ras-GRF2 in the hippocampus. Indeed, our previously published expression data of Ras-GRF1/2 (Fasano et al., 2009) and *in situ* hybridization data by the Allen Institute for Brain Science[4] indicate that Ras-GRF2 is abundantly expressed in the hippocampus and thus may vicariate for the absence of Ras-GRF1 in this brain structure.

In the near future it will be important to gain access to the behavioral data of both Ras-GRF2 KO and double Ras-GRF1 and 2 deficient mice, as well as from transgenic mice in which both Ras-GRF1 and 2 may be knocked down (via RNA interference) without altering the genome, in order to better understand the role of Ras-GRF1/2 dependent signaling in memory processing.

[4]http://www.brain-map.org

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ERK pathway activation bidirectionally affects visual recognition memory and synaptic plasticity in the perirhinal cortex

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ERK 1,2 pathway mediates experience-dependent gene transcription in neurons and several studies have identified its pivotal role in experience-dependent synaptic plasticity and in forms of long term memory involving hippocampus, amygdala, or striatum. The perirhinal cortex (PRHC) plays an essential role in familiarity-based object recognition memory. It is still unknown whether ERK activation in PRHC is necessary for recognition memory consolidation. Most important, it is unknown whether by modulating the gain of the ERK pathway it is possible to bidirectionally affect visual recognition memory and PRHC synaptic plasticity. We have first pharmacologically blocked ERK activation in the PRHC of adult mice and found that this was sufficient to impair long term recognition memory in a familiarity-based task, the object recognition task (ORT). We have then tested performance in the ORT in Ras-GRF1 knock-out (KO) mice, which exhibit a reduced activation of ERK by neuronal activity, and in ERK1 KO mice, which have an increased activation of ERK2 and exhibit enhanced striatal plasticity and striatal mediated memory. We found that Ras-GRF1 KO mice have normal short term memory but display a long term memory deficit; memory reconsolidation is also impaired. On the contrary, ERK1 KO mice exhibit a better performance than WT mice at 72 h retention interval, suggesting a longer lasting recognition memory. In parallel with behavioral data, LTD was strongly reduced and LTP was significantly smaller in PRHC slices from Ras-GRF1 KO than in WT mice while enhanced LTP and LTD were found in PRHC slices from ERK1 KO mice.

Keywords: ERK1,2, recognition memory, perirhinal cortex, synaptic plasticity

INTRODUCTION

Activation of synapse to nucleus signaling and regulation of gene transcription have been found to be crucial both for long term synaptic plasticity and memory consolidation (Davis and Squire, 1984; Mayford and Kandel, 1999; McGaugh, 2000; Abel and Lattal, 2001).

The ERK 1,2 (also called p42/44 mitogen-activated protein kinase) pathway mediates experience-dependent gene transcription and translational processes in neurons and several studies have identified its pivotal role in experience-dependent synaptic plasticity and in LTM consolidation involving cortex, hippocampus, amygdala, or striatum (Brambilla et al., 1997; Atkins et al., 1998; Blum et al., 1999; Mazzucchelli and Brambilla, 2000; Schafe et al., 2000; Di Cristo et al., 2001; Adams and Sweatt, 2002; Mazzucchelli et al., 2002; Cancedda et al., 2003; Kelleher et al., 2004; Thomas and Huganir, 2004; Doyère et al., 2007; Tsokas et al., 2007; Cohen-Matsliah et al., 2008; Leon et al., 2010; Lin et al., 2010; Dupont et al., 2011).

Many evidences indicate that the perirhinal cortex (PRHC) plays an essential role in familiarity-based object recognition (Suzuki et al., 1993; Zhu et al., 1995; Brown and Xiang, 1998; Murray and Bussey, 1999; Wan et al., 1999; Brown and Aggleton, 2001; Murray et al., 2007) and PRHC long term synaptic plasticity has been implicated in recognition memory (Warburton et al., 2003; Barker et al., 2006; Griffiths et al., 2008; Massey et al., 2008; Seoane et al., 2009).

Involvement of ERK pathway in recognition memory consolidation and reconsolidation has been suggested by two studies employing intraventricular or systemic administration of blockers of ERK1,2 activation (Kelly et al., 2003; Goeldner et al., 2008). It is still unknown whether ERK activation in PRHC is necessary for recognition memory consolidation and whether activation of the ERK pathway affects PRHC synaptic plasticity. Most important, it is unknown whether by modulating the gain of the ERK pathway it is possible to bidirectionally affect recognition memory and PRHC synaptic plasticity, not only causing an impairment by decreasing the gain, but also causing an enhancement by increasing it.

To assess the role of ERK activation in PRHC for recognition memory consolidation we performed a pharmacological block of ERK activation in the PRHC of adult mice and tested long term recognition memory in the object recognition task (ORT).

To assess the possibility that ERK can bidirectionally regulate visual recognition memory and PRHC plasticity we employed two mutant mouse lines. The first, Ras-GRF1 knock-out (KO) mice, has genetic deletion of Ras-GRF1, a guanine exchange factor which catalyzes the exchange of GDP for GTP on Ras, resulting in a reduced activation of ERK by neuronal activity (Brambilla et al., 1997; Fasano et al., 2009). The use of these mice allows to assess both the role of Ras-GRF1 signaling and the effects of reducing ERK activation in visual recognition memory and PRHC synaptic plasticity. The second, ERK1 KO mice (Mazzucchelli et al., 2002), has an increased activation of ERK2 in response to glutamate and exhibit enhanced striatal plasticity and striatal mediated memory. The use of these mice allows to assess both the relative role of ERK 1 and ERK 2 and the effects of increasing ERK 2 activation in visual recognition memory and in PRHC synaptic plasticity.

MATERIALS AND METHODS

ANIMALS

All mice used were aged between 3 and 6 months of age and were born and housed in our animal house. Lighting conditions were12 h light:12 h dark; animals were housed in standard laboratory cages, groups from five to seven animals per cage, according to the cage size and the animal house regulations. Housing and all experiments were performed in accordance with the Italian Ministry of Health guidelines for care and use of laboratory animals.

We have backcrossed both ERK1 (Mazzucchelli et al., 2002) and Ras-GRF1 KO (Brambilla et al., 1997) mice at least nine times in the C57BL6J mice line present in our animal house. Animals used for this experiments were at least F10 generation. Therefore, differences between the two transgenic lines cannot be ascribed to the genetic background, which is the same.

The breeding scheme for the ERK1 line was to use heterozygous male and female mice for breeding, so that in each litter there were homozygous pups for the deletion of ERK1 (ERK1 KO mice used in this study), heterozygous littermates, and wt littermates (ERK1 WT mice used in this study).

The breeding scheme for Ras-GRF1 KO mice, since this gene shows maternal imprinting (Brambilla et al., 1997) and only the paternal allele is expressed, was the following: we bred heterozygous males with WT females; half of the offsprings are wt (Ras-GRF1 WT used in this study) and half phenotypically KO (Ras-GRF1 KO used in this study).

We have used a total of 23 C57BL6J mice (13 males and 10 females) for the study on the effects of blocking ERK activation in the PRHC on recognition memory (16 mice, 9 males and 7 females) and for the immunohistochemistry of phospho-ERK (pERK; seven mice, four males and three females).

We have used a total of 100 Ras-GRF1 KO mice (54 males and 46 females) for the behavioral tests, 32 (20 males and 12 females) for the ORT test at 1 and 12 h interval and 68 (34 males and 34 females) for the reconsolidation experiments. As controls, 74 Ras-GRF1 WT mice have been used (41 males and 33 females), 29 (15 males and 14 females) for the ORT test at 1 and 12 h interval and 45 (26 males and 19 females) for the reconsolidation experiments. In addition, 20 Ras-GRF1 KO mice (12 males, 8 females) and 18 Ras-GRF1 WT mice (11 males, 7 females) have been used for the electrophysiology.

A total of 52 ERK1 KO mice have been used (22 females, 30 males), 23 for the behavioral experiments (14 males and 9 females), 18 for the electrophysiology (11 males, 7 females), and 11 for biochemistry (6 females and 5 males); as control, 47 ERK1 WT mice have been used (25 males, 22 females), 21 for the behavioral studies (11 males and 10 females), 16 for the electrophysiology (9 males, 7 females), and 10 for biochemistry (5 females and 5 males).

OBJECT RECOGNITION TASK

The apparatus consisted of a square arena (60 cm × 60 cm × 30 cm) constructed in PVC with black walls and white floor. The objects were either three-dimensional metal, plastic, glass, or china objects of different shapes, or cubes (15 cm wide) made of transparent Plexiglas that differed for the visual patterns lining the walls. Box and objects were cleaned up between trials to stop the build-up of olfactory cues (De Rosa et al., 2005).

The experimental protocol was modified from Ennaceur and Delacour (1988). Briefly, mice received one session of 5 min duration in the empty arena to help them habituate to the apparatus and test room (habituation phase). Twenty-four hours later, each mouse was placed in the arena and exposed to two identical objects (sample phase) for 5 min and returned to its cage. After a variable delay (1, 12, 48, 72, or 96 h), mice were placed back in the arena and exposed to a familiar object (object identical to those in sample phase) and to a novel object for 5 min (test phase). Objects were placed in the same locations as in sample phase. Time spent exploring each object was recorded for each animal and for each condition and a discrimination index was calculated:

$$\text{Discrimination Index} = \frac{\begin{array}{c}[(\text{Exploration time of New object})\\ -(\text{Exploration time of Old object})]\end{array}}{\begin{array}{c}[(\text{Exploration time of New object})\\ +(\text{Exploration time of Old object})]\end{array}}.$$

Exploration of an object was defined as directing the nose to the object at a distance of ≤3 cm and/or touching it with the nose or forepaws; time spent turning around the object was not included.

We have used in this study pairs of objects previously validated by us in a large group of wt and mutant animals for not giving differential exploration times. For each retention time interval, the new object for half of the animals, wt, or mutants, was the familiar object for the other half; also the position of the novel and the familiar object (left–right) was balanced. When the same animals performed more than one ORT test, different pairs of objects were used. When different groups of animals performed the same ORT test we used the same pairs of objects.

Reconsolidation protocol

Mice were first habituated to the open arena in the absence of objects for 20 min a day for two consecutive days ("habituation phase"). Twenty-four hours after the second habituation phase, mice were reintroduced in the arena and exposed to two identical objects for either a single familiarization trial of 5 min duration (single sample phase) or eight familiarization trials, duration 5 min each and distributed in four blocks of two sessions each (multiple

sample phase); the interval between blocks was 90 min and the interval between sessions within each block was 5 min. During the intersession intervals mice were placed into an holding cage in the experimental room.

Forty-eight hours after the single or the multiple sample phase mice were exposed to a familiar object (identical to those in the sample phase) and to a novel object for 5 min ("test phase"). To test reconsolidation, 24 h after the multiple sample phase mice were exposed again to the same two sample objects explored during the sample phase for a single 5 min period to reactivate the memory trace (reactivation phase). Memory retention after reactivation was tested with novel and familiar objects (test phase) either 10 min, to test post-reactivation short term memory (PR-STM), or 24 h later, to test post-reactivation long term memory (PR-LTM).

CANNULAE IMPLANTATION AND DRUG DELIVERY

Surgery

Mice were anesthetized (avertin 2 ml/100 g), placed in a stereotaxic apparatus and implanted bilaterally with 22 gage indwelling guide cannulae according to the following procedure: two holes were made in the skull through which the guide cannulae were inserted, perpendicular to the horizontal plane, according to the following coordinates: AP −3.05 mm (relative to bregma), L ±4.55 mm (relative to midline), V −1.5 mm (relative to surface of the brain). Cannulae were then fixed to the skull with dental cement. In order to prevent accidental blockage and keep the guide cannulae clear, obdurators cut to extend 1.1 mm beyond the tip of the guide cannulas and with an outer diameter of 0.36 mm were inserted into the guides and remained there except during infusions. Animals were allowed to recover from the surgery for at least a week before the beginning of behavioral testing.

Drug delivery

Immediately after the ORT's sample phase animals were gently restrained by the experimenter and the infusion process was started. The obdurators were removed, and the 28 gage infusion cannulas, which were cut to extend 1 mm beyond the tip of the guides, were inserted into the guides. ERK inhibitor UO126 (5 mM in 50% DMSO) or vehicle (saline in 50% DMSO) was injected bilaterally (0.5 μl on each side given over 2 min) by means of a 25-μl Hamilton syringe; the infusion cannula was left for an additional minute to allow the full deployment of the drug. The whole process required a total of 10 min for each animal, approximately. Recognition memory was assessed in the test phase 12 h later. Administration of the drug immediately after the sample phase (and not before) should ensure that it affects memory consolidation rather than information encoding (Winters and Bussey, 2005).

IMMUNOHISTOCHEMICAL PROCEDURES

Phospho-ERK immunostaining

Thirty minutes after learning mice were sacrificed and transcardial perfusion was executed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were quickly removed, post-fixed for 12 h and cryoprotected in 30% sucrose overnight, and 40 μm coronal sections were cut on cryostat and processed for pERK immunohistochemistry. Free-floating sections were subjected to a 2-h block (PBS containing 10% BSA and 0.4% Triton X-100 at room temperature) followed by overnight incubation with pERK monoclonal antibody (1:1000, Sigma, St. Louis, MO, USA; in PBS containing 1% BSA and 0.1% Triton X-100, at 4°C). pERK was revealed using Alexa 568 labeled secondary antibodies (1:200 Vector Laboratories, Burlingame, CA, USA; diluted in 1% BSA and 0.4% Triton X-100 in PBS). Slices were coded and, for each animal, confocal images (Olympus FV-300) of at least three representative fields (706 μm × 706 μm) for each PRHC were acquired.

After immunohistochemistry for pERK, the staining of inhibitory cells was performed using 1:500 mouse anti GAD67 (Chemicon, USA) monoclonal primary antibody (4°C overnight) followed by 1:200 goat anti-mouse secondary antibody conjugated to Alexa Fluor 488 fluorophore.

WESTERN BLOTTING

After decapitation, brains were removed rapidly and frozen on dry ice. A cortical area corresponding to PRHC was bilaterally dissected and homogenized in lysis buffer.

Tissue homogenates were centrifuged at 13000 *g* for 30 min at 4°C. The supernatant (cytoplasmic fraction) was aspirated, and protein concentration was determined in each sample by the Bradford method (Bio-Rad, Milan, Italy). Each sample was boiled, and 25 μg/lane of protein was loaded into 12% acrylamide gels using the Precast Gel System (Bio-Rad). Samples were blotted onto nitrocellulose membrane (Amersham, Bucks, UK). Membranes were blocked in 4% BSA in Tris-buffered saline for 1 h and then incubated overnight at 4°C with antibodies against either Phospho-p42/44 Map Kinase (1:1000, Sigma) or α-Tubulin (1:5000, Sigma). Blots were then rinsed in TTBS and TBS, incubated for 1 h in HRP-conjugated anti-mouse (1:3000 Bio-rad, Italy, in 2% BSA and TTBS), rinsed, incubated in enhanced chemiluminescent substrate (Bio-rad, Italy) and acquired by ChemiDoc (Bio-rad). The immunoblots were analyzed with ImageJ software to measure the optical density of the bands, using α-tubulin as loading control. To minimize variability each sample was loaded in parallel in two lanes and two gels were run simultaneously on the same apparatus.

IN VITRO ELECTROPHYSIOLOGY

Slices of the perirhinal and surrounding cortex were prepared from adult (P60–120) mutant mice and their WT littermates. All efforts were made to minimize animal suffering and the number of animals. All experiments were performed in accordance with the Italian Ministry of Health guidelines for care and use of laboratory animals. Animals were anesthetized by isoflurane inhalation and decapitated. The brain was rapidly removed and immersed in ice-cold modified artificial cerebrospinal fluid (composition in mM: NaCl, 132.80; KCl, 3.10; HEPES, 10.00; NaHCO3, 4.00; ascorbic acid, 1.00; myo inositol, 0.50; sodium pyruvate, 2.00; K2HPO4, 1.00; glucose, 5.00; MgCl2, 2.00; and CaCl2, 1.00) bubbled with oxygen (Di Cristo et al., 2001).

A mid-sagittal section was made through the brain, the rostral and caudal parts of the brain were removed by single scalpel cuts made at approximately 45° to the dorsoventral axis (Massey

et al., 2001), and each remaining half was glued by its rostral end onto a vibroslice stage Leica VT 1000S microslicer (Leica Microsystems; Nussloch, Germany). Slices (340 μm thick) which included the perirhinal, entorhinal, and temporal cortices were cut and allowed to recover for at least 1 h at 30°C in a submersion chamber containing oxygenated modified artificial cerebrospinal fluid.

A single slice was placed in a submerged recording chamber filled with artificial cerebrospinal fluid (composition in mM 132.80 NaCl, 3.10 KCl, 1.00 K2HPO4, 10.00 HEPES, 1.00 ascorbic acid, 0.50 myo inositol, 2.00 sodium pyruvate, 4.00 NaHCO3, 1 MgCl2, 2 CaCl2, and 5.00 glucose), 30–31°C, flow rate, 1.5 ml/min.

Standard extracellular recording electrodes (1.0 MΩ glass electrodes filled with artificial cerebrospinal fluid) were approximately positioned in layer II, beneath the rhinal fissure, and were used to record field potentials. A stimulating tungsten bipolar electrode was placed in intermediate layers II/III, 0.16–0.36 mm laterally with respect to the recording electrode. Pulses of 100 μs duration were delivered every 30 s (0.033 Hz) to the stimulating electrode. Recorded field potentials were filtered between 0.1 and 3 kHz, amplified 1000 times (Axon CyberAmp), sampled at 10 kHz and fed to a computer using LabVIEW software (National instruments, USA). Stimulation intensity was adjusted to evoke a field potential amplitude 50% of the maximal value. After 15 min of stable baseline obtained with the 0.033-Hz stimulation, either TBS to evoke LTP or charbacol administration to evoke LTD (Massey et al., 2001) were delivered.

Theta burst stimulation protocol consisted in four repetitions, 15 s apart, of 10 trains of 4 pulses at 100 Hz, with an intertrain interval of 200 ms. Field potential amplitudes were recorded every 30 s for 60 min following the cessation of the TBS stimulation protocol. Data from different slices were normalized to baseline amplitudes, averaged, and reported as means ± SEM as a function of time. To assess the final level of potentiation, the mean normalized EPSP amplitude over the last 15′ of recordings was used.

Carbachol (50 μM, Sigma) was applied for 10′ (Massey et al., 2001). Field potential amplitudes were recorded every 30 s for 60 min following the initiation of Carbachol application. Data from different slices were normalized to baseline amplitudes, averaged, and reported as means ± SEM as a function of time. To assess the final level of depression, the mean normalized EPSP amplitude over the last 20′ of recordings was used.

STATISTICS

All data were analyzed with SigmaStat statistical package. Differences in object exploration times between KO and WT mice were analyzed with the unpaired Student's *t*-test or Mann–Whitney *U*-test, depending to the result of the test for Normal distribution of the data. Differences between the exploration time of the new and that of the familiar object in the test phase was analyzed, for each group of KO or WT mice, with the paired Student's *t*-test or the Wilcoxon Signed Rank test, depending to the result of the test for Normal distribution of the data. Differences between the discrimination indexes were analyzed with the Wilcoxon Signed Rank test (for paired groups) or the Mann–Whitney *U*-test (for unpaired groups).

Field potential amplitudes in LTP and LTD experiments were analyzed by means of repeated measures analysis of variance and appropriate *post hoc* analysis. *p* Values less than 0.05 were taken to indicate statistically significant differences.

RESULTS

ACTIVATION OF ERK IN THE PRHC IS NECESSARY FOR RECOGNITION MEMORY CONSOLIDATION

To assess whether activation of the MAP kinase pathway in the PRHC is necessary for visual recognition memory consolidation, we injected into the PRHC an inhibitor of MEK (the ERK activating kinase) UO126, or vehicle for control, immediately after (3 min) the sample (learning) phase of the ORT. UO126 (5 mM in 50% DMSO) or vehicle (saline in 50% DMSO) were injected (0.5 μl) bilaterally through guide cannulae previously implanted into the PRHC (**Figures 1A,B**).

To control that U0126 injections were effective in blocking ERK activation, we performed an immunohistochemistry for pERK 30′ after the sample phase in animals ($n = 3$) injected with U0126 in one PRHC and with vehicle in the contralateral PRHC. We found that pERK immunopositive cells were clearly present in the PRHC treated with vehicle but were significantly reduced from the cortex treated with U0126, showing that U0126 effectively diffused from the injection cannula and blocked ERK activation (**Figure 1C**). pERK was localized in the superficial and deep layers of the PRHC (**Figure 1D**). In the visual cortex, pERK immunostaining after visual stimulation is present mainly in excitatory neurons (Cancedda et al., 2003) and evident in dendrites and cell bodies (Boggio et al., 2007); a similar pattern of dendritic and somatic pERK immunostaining was found in the PRHC (**Figures 1E,F**).

A total of 16 C57BL/6J mice was employed and each animal performed the ORT twice, with an interval of at least 3 days between the two tests. For both ORT trials, the retention interval between the sample phase and the test phase was 12 h. Half of the animals received vehicle injection during the first ORT and U0126 injections during the second one. The other half of the animals was injected with U0126 during the first ORT and with vehicle during the second one. There was no difference between the total exploration time during sample phase in the second ORT trial between mice treated with U0126 (30.83 ± 7.42 s) at the first ORT trial and those treated with vehicle (25.75 ± 6.06 s; $p = 0.222$; paired *t*-test). Also total object exploration time during the test phase did not differ between vehicle (28.73 ± 3.09 s) and UO126 treated mice (24.23 ± 5.25 s), ($p = 0.234$, paired *t*-test). This suggests that blockade of ERK activity has no effect on exploratory behavior and locomotor activity.

In the test phase, mice injected with vehicle showed the expected preferential exploration of the novel object (**Figure 1G**, paired *t*-test, exploration time of the novel versus the familiar object $p = 0.029$) while mice injected with U0126 did not (**Figure 1G**, paired *t*-test, exploration time of the novel versus the familiar object $p = 0.303$). As a consequence, the discrimination index in the group treated with UO126 was significantly lower than in the group treated with vehicle (Wilcoxon Signed Rank test, $p = 0.011$; **Figure 1G**).

These data suggest that activation of ERK pathway in the PRHC is necessary for consolidation of long term recognition memory.

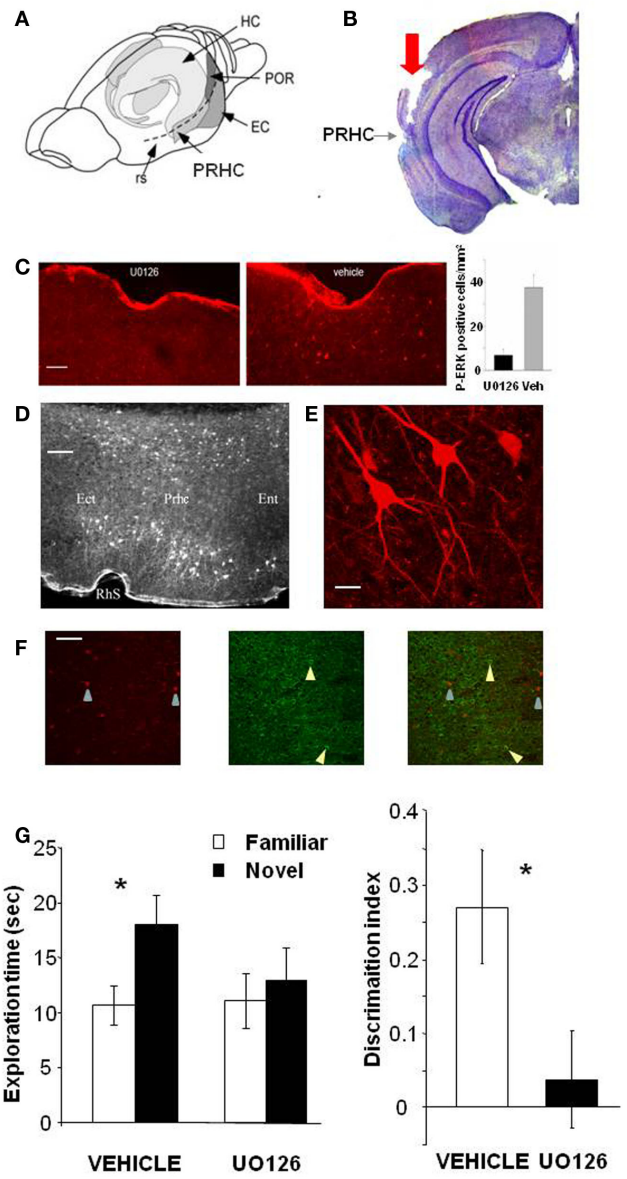

FIGURE 1 | Activation of ERK in the Perirhinal Cortex is necessary for recognition memory consolidation. (A) Location of the perirhinal cortex (PRHC) in a schematic lateral view of the mouse brain. Shading indicates the location of the hippocampal formation (HC) and the perirhinal (PRHC), enthorinal (EC), and postrhinal cortices (POR); *rs* indicates the rhinal sulcus. **(B)** Photomicrograph of a coronal brain section showing the track (indicated by red arrow) left by a cannula inserted into the PRHC. The PRHC is also indicated. **(C)** U0126 infusion is effective in blocking ERK activation in the perirhinal cortex. Example of immunohistochemistry for phospho-ERK 30′ after the sample phase in one animal injected with U0126 in one PRHC and with vehicle in the contralateral PRHC. Phospho-ERK immunopositive cells are clearly present in the PRHC treated with vehicle (right) but are absent from the cortex treated with U0126 (left), showing that U0126 effectively diffused from the injection cannula and blocked ERK activation in the PRHC. In the inset, the mean number of phospho-ERK immunopositive cells per square millimeter counted in the U0126 treated PRHC (7 ± 2.6) and in the vehicle treated PRHC (38 ± 5.7; $n = 4$ animals) is reported. The difference between U0126 and vehicle treated side is significant (paired *t*-test,

(Continued)

FIGURE 1 | Continued
$p = 0.035$). Calibration bar: 50 μm. **(D)** Localization of cells immunopositive for phospho-ERK 30′ after exploration of the ORT arena with two new objects. Ect, ectorhinal cortex; Prhc, perirhinal cortex; Ent, entorhinal cortex; RhS, rhinal sulcus. Calibration bar 100 μm. **(E)** High power image of phospho-ERK immunopositive neurons in the perirhinal cortex. Calibration bar 15 μm. **(F)** Example of a double staining for phospho-ERK and GAD 67 in the perirhinal cortex. Left, cells immunopositive for phospho-ERK in the PRHC. Arrows point to two stained neurons; center, immunostaining for GAD67. Arrows point to two stained neurons; right, merge of the two images. Calibration bar 80 μm. **(G)** Perirhinal focal infusion of MAPK blocker UO126 impairs long term (12 h) object recognition memory. UO126 (5 mM in 50% DMSO, $n = 16$) or vehicle (saline in 50% DMSO, $n = 16$) were injected (0.5 μl bilaterally) immediately (3 min) after the sample phase of the ORT. Recognition memory was tested (test phase) 12 h after the sample phase. Left: Exploration of novel compared to familiar objects during the test phase. Vehicle injected mice explore the novel object significantly more than the familiar object (asterisk, $p < 0.05$, paired *t*-test) while UO126 injected mice do not (paired *t*-test, $p > 0.05$). Right: discrimination index in the test phase for vehicle and U0126 injected mice. The latter exhibit a significantly lower discrimination index with respect to vehicle injected mice (Wilcoxon Signed Rank test, $p < 0.05$, asterisk).

RAS-GRF1 KO MICE SHOW DEFICITS IN CONSOLIDATION OF VISUAL RECOGNITION MEMORY

ERK1 and ERK2 activation is the result of the integration of multiple signals deriving from neuronal activity and neurotrophin signaling, such as Ca^{2+} influx, activation of G-protein coupled receptors, and trk receptor signaling. Central to ERK activation is the increase in the active, GTP bound form of the small G-Protein Ras, which causes MEK phosphorylation. Ras activation, in its turn, is under control of guanine exchange factors, GEF, which catalyze the exchange of GDP for GTP on Ras, such as the Sos and the Ras-GRF families. Ras-GRFs activate Ras in response to Ca^{2+} signaling and muscarinic receptor activation (Farnsworth et al., 1995; Mattingly and Macara, 1996).To investigate the specific role of Ras-GRF1 signaling and the effects of reducing only the component of ERK activation resulting by Ras-GRF1 on recognition memory, we evaluated the performance in the ORT of Ras-GRF1 KO mice ($n = 32$) compared to their WT littermates ($n = 29$).

One group of Ras-GRF1 KO ($n = 15$) and Ras-GRF1 WT ($n = 14$) mice performed the ORT with a retention interval of 1 h; a second group of Ras-GRF1 KO ($n = 17$) and Ras-GRF1 WT mice ($n = 15$) performed the ORT with a retention interval of 12 h. Total object exploration time during the sample phase was never significantly different between Ras-GRF1 KO and WT mice.

In WT mice, preferential exploration of the novel object during the test phase is present at 1 and 12 h after familiarization (paired *t*-test, $p < 0.001$ at 1 h and $p < 0.01$ at 12 h; **Figure 2A**); in KO mice preferential exploration of the novel object during the test phase is present only 1 h after familiarization (paired *t*-test, $p < 0.001$ at 1 h interval, $p = 0.690$ at 12 h; **Figure 2A**), showing a lack of long term retention.

For both Ras-GRF1 KO and WT mice the discrimination index significantly decreases with increasing retention interval (discrimination index at 12 h significantly lower than at 1 h, $p < 0.001$ both for WT and Ras-GRF1 KO mice). However, the decrease is faster for KO mice; indeed, the discrimination index of KO mice is significantly lower compared with WT mice after the retention interval

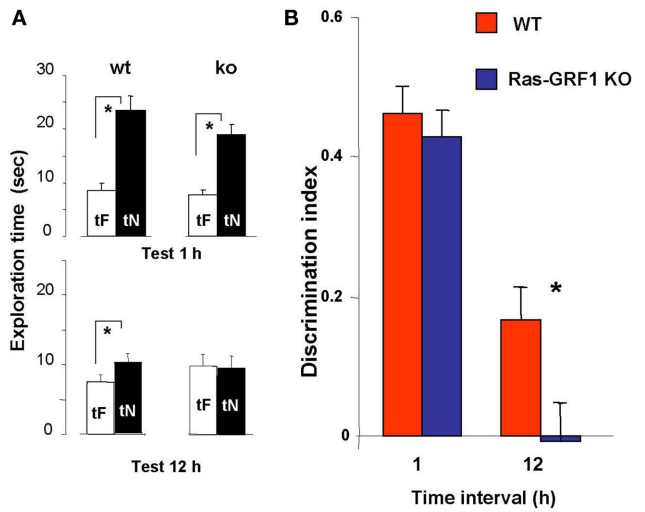

FIGURE 2 | Ras-GRF1 KO mice show long term visual recognition memory deficits. (A) Mean exploration times for the 1- and 12-h interval experiment in Ras-GRF1 KO mice and their WT littermates. The exploration time of the familiar object, tF, and the exploration time of the novel object, tN, in the test phase significantly differ (asterisk) for WT mice both at 1 h ($n = 14$) and at 12 h interval ($n = 15$; paired *t*-test, $p < 0.001$ at 1 h and $p < 0.01$ at 12 h) but for KO mice only at 1 h ($n = 15$) there is a significant difference (paired *t*-test, $p < 0.001$). At 12 h KO mice ($n = 17$) do not show any differential exploration of the new with respect to the familiar object (paired *t*-test, $p = 0.69$), suggesting a consolidation deficit. **(B)** Memory retention curve for ORT in Ras-GRF1 KO mice and their WT littermates. Discrimination index is plotted against time interval between familiarization and test. Asterisk denotes significant difference between WT and KO mice (two-way ANOVA, time × genotype, *post hoc* Holm–Sidak method).

of 12 h ($p < 0.023$; two-way ANOVA, time × genotype, *post hoc* Holm–Sidak method; **Figure 2B**).

Thus Ras-GRF1 KO mice have normal memory at 1 h retention interval but exhibit a deficit at 12 h, suggesting that lack of Ras-GRF1 activation of the ERK pathway results in a defect in recognition memory consolidation.

RAS-GRF1 KO MICE HAVE DEFICITS IN VISUAL RECOGNITION MEMORY RECONSOLIDATION

According to the memory reconsolidation hypothesis each time a memory is reactivated/retrieved it becomes labile and must undergo a process of consolidation to be maintained to further long term (see Lee, 2009; Nader and Hardt, 2009). We examined then the possibility that consolidated visual recognition memory traces, after being reactivated by re-exposure to the learning situation, undergo a process of reconsolidation requiring Ras-GRF1.

The consolidation deficit exhibited by Ras-GRF1 KO mice might represent a difficulty, as determining the role of Ras-GRF1 in reconsolidation after retrieval requires that mice form a consolidated long term memory of the objects. However, it has been shown that deficits in visual recognition memory consolidation can be overcome by extended and distributed training in zif 268 KO mice (Bozon et al., 2003). Thus, we first examined whether Ras-GRF1 KO mice could form a long term visual recognition memory if given additional exposures to the objects in a distributed training paradigm. To do so, we compared the performance in the test phase at 48 h after the sample phase in two groups of KO mice, one ($n = 20$) undergoing the ORT procedure previously employed (one familiarization trial of 5 min during the sample phase) and one ($n = 16$) receiving extended training, eight familiarization sessions of 5 min each during the sample phase. The eight familiarization sessions were distributed in four blocks of two sessions each; the interval between blocks was 90 min and the interval between sessions within each block was 5 min (**Figure 3A**). We found that the discrimination index for the group exposed to the extended training was significantly greater than that of the group exposed to a single familiarization session (Mann–Whitney, $p = 0.012$; **Figure 3B**). Preferential exploration of the novel object during the test phase was clearly present in Ras-GRF1 KO mice exposed to eight familiarizations (paired *t*-test, $p < 0.001$) while it was absent in the group not exposed to extended training ($p = 0.252$). The discrimination index of Ras-GRF1 KO mice subjected to extended training did not differ from that of Ras-GRF1 WT mice subjected to the same extended training (**Figure 3C**; $p = 0.494$ Mann–Whitney). These data indicate that extended training can counteract memory consolidation deficits in Ras-GRF1 KO mice.

Being able to provide both genotypes with long lasting memories, we evaluated the effect of memory reactivation on PR-LTM traces. To do so, mice of both genotypes were provided with the extended training during the sample phase and then, 48 h later, performance in the test phase was assessed with or without intervening memory reactivation, performed 24 h after the sample phase by re-exposing the animals for 5 min to the same objects used in the sample phase (**Figure 3A**).

As shown in **Figure 3C**, memory reactivation did not affect subsequent PR-LTM in WT mice (**Figure 3C**): the discrimination index in the test phase with (0.241 ± 0.064 $n = 16$) or without (0.214 ± 0.093, $n = 20$) memory reactivation did not differ, (Mann–Whitney, $p = 0.430$). In KO mice memory reactivation resulted in a significant reduction of the discrimination index, compared to the condition without reactivation (discrimination index with reactivation 0.062 ± 0.076, $n = 17$; without reactivation 0.302 ± 0.1, $n = 15$, Mann–Whitney, $p = 0.026$; **Figure 3C**). Accordingly, preferential exploration of the novel object during the test phase for Ras-GRF1 KO mice was only present if memory had not been reactivated (exploration time of new versus familiar object, paired *t*-test, $p < 0.001$ in the condition of no reactivation and $p = 0.212$ in the condition of reactivation; **Figure 3D**), suggesting that reactivation had canceled the visual recognition memory trace.

In order to ascribe the deficit shown by Ras-GRF1 KO mice 24 h after reactivation to a defect in memory consolidation rather then to an aspecific effect of reactivation we assessed PR-STM. The experimental protocol for the sample and the reactivation phase was as described above but in this case visual recognition memory was assessed 10 min after reactivation. We found that discrimination index in KO and WT mice was not significantly different (Mann–Whitney, $p = 0.427$; **Figure 3E**), suggesting that reactivation does not affect PR-STM of KO mice.

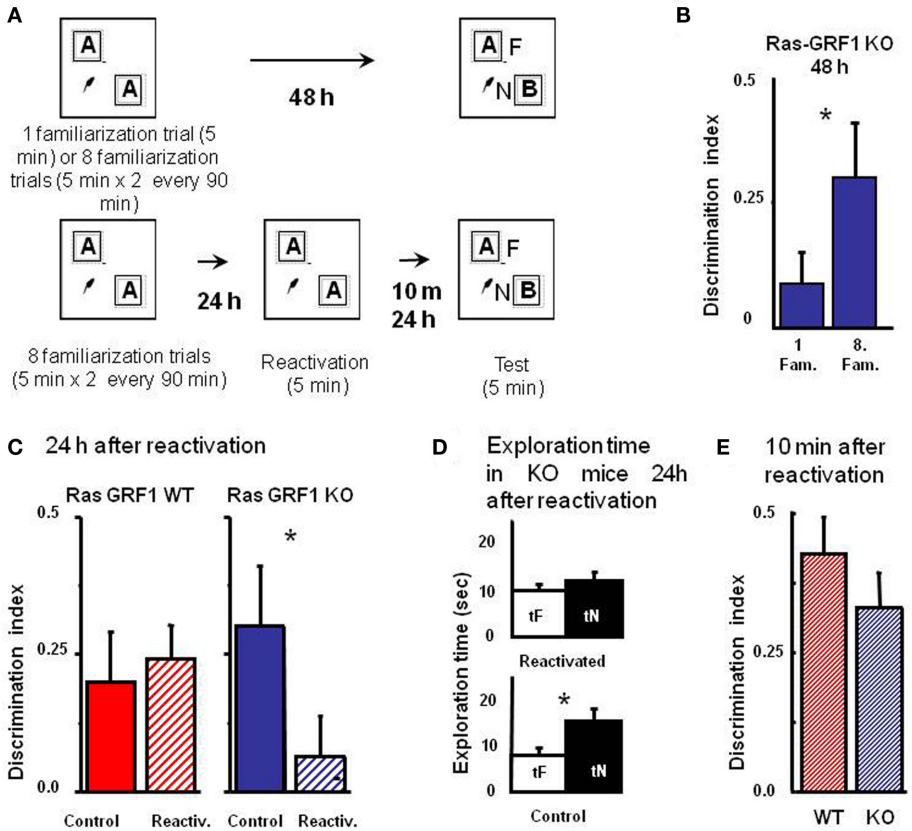

FIGURE 3 | Ras-GRF1 KO mice exhibit a reconsolidation deficit. (A) Protocols for repeated familiarizations (top) and for memory reconsolidation test (bottom). Top: mice are introduced into the arena in the presence of two identical objects for one (single familiarization phase) or eight consecutive sessions of 5 min (multiple familiarization phase). After a delay of 48 h the test phase is performed. Bottom: mice are introduced into the arena in presence of two identical objects for eight consecutive sessions of 5 min each. Twenty-four hours later mice memory trace is reactivated by re-exposing the mice to the same two familiar objects. After a delay of 10 min or of 24 h the test phase is performed. **(B)** Discrimination index of Ras-GRF1 KO mice 48 h after a single familiarization session or after repeated familiarization sessions. Asterisk denotes significant difference between Ras-GRF1 KO mice ($n = 16$ for the repeated familiarization, $n = 20$ for the single familiarization) in the two conditions (Mann–Whitney $p = 0.012$). Repeated exposure to the stimuli is sufficient to compensate for the deficit of visual recognition memory of Ras-GRF1 KO mice. **(C)** Discrimination index of Ras-GRF1 KO and WT mice 48 h after repeated familiarization sessions and 24 h after memory reactivation. In wt, re-exposure of the animals to the stimuli did not interfere with recognition of the familiar stimulus 24 h later (control $n = 20$, reactivated $n = 16$; $p = 0.43$ Mann–Whitney). By contrast a single exposure to the familiar stimulus 24 h after its memorization makes the memory trace labile in Ras-GRF1 KO mice: 24 h after reactivation, discrimination index was significantly decreased with respect to that found without reactivation (control $n = 15$, reactivated $n = 17$; $p = 0.026$ Mann–Whitney). **(D)** Exploration time of novel and familiar object of Ras-GRF1 KO mice 24 h after reactivation: there is no preferential exploration of the novel object (time of exploration of new object, tN versus time exploration familiar object, tF, control $p < 0.001$; reactivated $p = 0.212$ paired t-test). Asterisks denote significant differences. **(E)** Discrimination index 10 min after reactivation; the deficit in Ras-GRF1 KO mice was not present 10 min after reactivation.

These findings suggest the presence of a deficit in memory reconsolidation in Ras-GRF1 KO mice, and imply a critical role for ERK activation by the Ras-GRF1 pathway in this process.

ERK1 KO MICE HAVE A LONGER-LASTING VISUAL RECOGNITION MEMORY

To investigate the effects of an enhancement of ERK activation on visual recognition memory, we evaluated the memory performance in a group of 23 ERK1 KO mice compared to their littermates WT ($n = 21$). ERK1 KO mice present an enhanced activation of ERK2 by glutamate as assessed *in vitro* (Mazzucchelli et al., 2002). Recently, it has been shown that ERK2 phosphorylation is enhanced *in vivo* in dorsal hippocampus of ERK1 KO mice with respect to WT mice (Tronson et al., 2008). In line with these results, we found a significantly higher level of ERK2 phosphorylation in the PRHC of ERK1 KO ($n = 11$) with respect to WT mice ($n = 10$, t-test, $p = 0.039$; **Figure 4A**).

To assess the time span of retention in the ORT, each animal performed the ORT with a different retention intervals between sample and test phase. For each ORT session, mice went through a new sample phase with two equal objects never used before and then a test phase after the appropriate time interval.

We found that discrimination index in ERK1 KO mice did not differ from that of ERK1 WT mice at 1 or 48 h retention interval but was significantly higher than that of WT for the retention interval of 72 h (two-way ANOVA for repeated measures, time × genotype, genotype, time, and interaction all significant $p < 0.001$, *post hoc* Tukey's test; **Figure 4B**). In KO mice, but

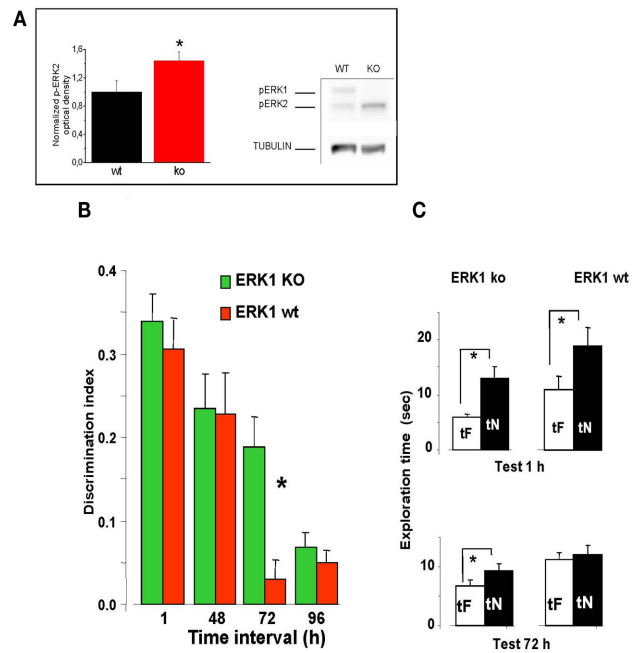

FIGURE 4 | Longer lasting visual recognition memory in ERK1 KO mice. (A) Left, quantification of phospho-ERK2 optical density, normalized to tubulin, in the perirhinal cortex of ERK1 KO ($n = 11$) and ERK1 WT ($n = 10$) mice. The difference is statistically significant (*t*-test, $p = 0.039$). Right, example of immunoblotting. **(B)** Memory retention curve for ORT in ERK1 KO mice ($n = 23$) and their WT littermates ($n = 21$). Discrimination index is plotted against time interval between familiarization and test. Asterisks denote significant difference between WT and KO mice (two-way ANOVA for repeated measures, time × genotype, genotype, time, and interaction all significant $p < 0.001$, *post hoc* Tukey's test). Performance differs at 72 h. **(C)** Exploration times in the ORT for ERK1 KO mice ($n = 23$) and their WT littermates ($n = 21$). At 1 and 72 h intervals. Asterisks denote significant difference between tF and tN. At 1 h there is a significant difference for both groups but at 72 h only KO mice show a differential exploration of the new with respect to the familiar object (paired *t*-test, $p < 0.001$ for KO and $p < 0.112$ for WT mice).

not in WT, preferential exploration of the novel object during the test phase is found up to 72 h from sample phase (paired *t*-test, exploration time of novel versus familiar object at 72 h $p < 0.001$ for KO mice and $p = 0.112$ for WT mice; **Figure 4C**), indicating that the memory trace persisted for a longer in time. Thus ERK1 KO mice show a longer lasting visual recognition memory.

GENETIC MODULATION OF ERK2 ACTIVATION BIDIRECTIONALLY AFFECTS SYNAPTIC PLASTICITY IN THE PRHC

In the previous sections we have shown that the performance in a visual recognition memory task can be enhanced or impaired by genetically interfering with ERK pathway activity. To assess whether the behavioral phenotype of Ras-GRF1 and ERK1 KO was associated with an alteration of synaptic plasticity in the PRHC, we studied LTP and LTD in PRHC of mice belonging to both genetically mutated groups.

LTP was induced in layer II/III horizontal connections using a standard TBS protocol. We found a stable and persistent LTP in all the three mice groups, WT, (littermates of ERK1 KO, and Ras-GRF1 KO, pooled together since there was no statistical difference between the two), ERK1 KO, Ras-GRF1 KO (**Figure 5**). However, in accordance with the hypothesis of a dependence of synaptic plasticity from genetic modulation of ERK pathway activation, we found that layer II/III PRHC LTP was significantly lower in Ras-GRF1 KO mice [median increase 107.7% of baseline (interquartile ranges 96–115), $n = 12$] than WT [median increase 136% of baseline (interquartiles 119–149), $n = 21$], while LTP induced in ERK1 KO [median increase 155.8% ± of baseline (interquartiles 149–169), $n = 7$] was significantly higher compared with WT (ANOVA, genotype × time, factor genotype: $p < 0.001$, all three groups differ one from each other, *post hoc* Student–Newman–Keuls Method).

In another group of animals from the three genotypes LTD was pharmacologically induced by 10 min administration of 50 μM carbachol as in Massey et al. (2001). We found that Ras-GRF1 mutants had a significantly impaired LTD (95.5 ± 3.8% of baseline, $n = 8$) while ERK1 mutants showed a significantly enhanced LTD (70 ± 3.3% of baseline, $n = 11$) when compared with WT animals, littermates of ERK1KO and Ras-GRF1 KO, (80.75 ± 3% of baseline, $n = 13$; ANOVA, genotype × time, factor genotype $p < 0.001$, all three groups differ one from each other, *post hoc* Holm–Sidak Method, $p < 0.050$).

Thus, synaptic plasticity in PRHC can be bidirectionally regulated by genetic manipulating the activation of the ERK pathway. The differences found in PRHC synaptic plasticity of Ras-GRF1 and ERK1 KO mice reflect the differences in behavioral performance observed in the visual recognition memory task: reduced LTP and strongly impaired LTD in Ras-GRF1 KO mice, which have a reduced ERK activation; enhanced LTP and LTD in ERK1 KO mice, which have enhanced ERK2 activation.

DISCUSSION

ERK ACTIVATION IN THE PRHC IS NECESSARY FOR LONG TERM RECOGNITION MEMORY

ERK involvement in recognition memory had been already suggested by Kelly et al. (2003), showing that intraventricular injection before the ORT learning phase of U0126, a blocker of the ERK kinase MEK, impaired consolidation and reconsolidation of recognition memory. This has been subsequently confirmed by Goeldner et al. (2008), employing systemic treatment with SL327, another MEK blocker. These two papers do not allow to assess the role of ERK activation in the PRHC in recognition memory consolidation, since the treatment with blockers of ERK activation was not specifically directed to the PRHC.

Our results show that it is sufficient to block ERK activation in the PRHC after the learning phase to impair long term recognition memory in the ORT. This suggests that ERK activation in the PRHC is necessary for recognition memory consolidation. It also suggests that ERK activation in other medial temporal lobe structures possibly involved in recognition memory, such as the hippocampus, (see Kelly et al., 2003) is not sufficient to mediate the formation of a long term recognition memory. This strengthens the importance of the PRHC and of the plasticity processes triggered within its circuits by learning in the formation of long term, familiarity-based, recognition memory (Warburton et al.,

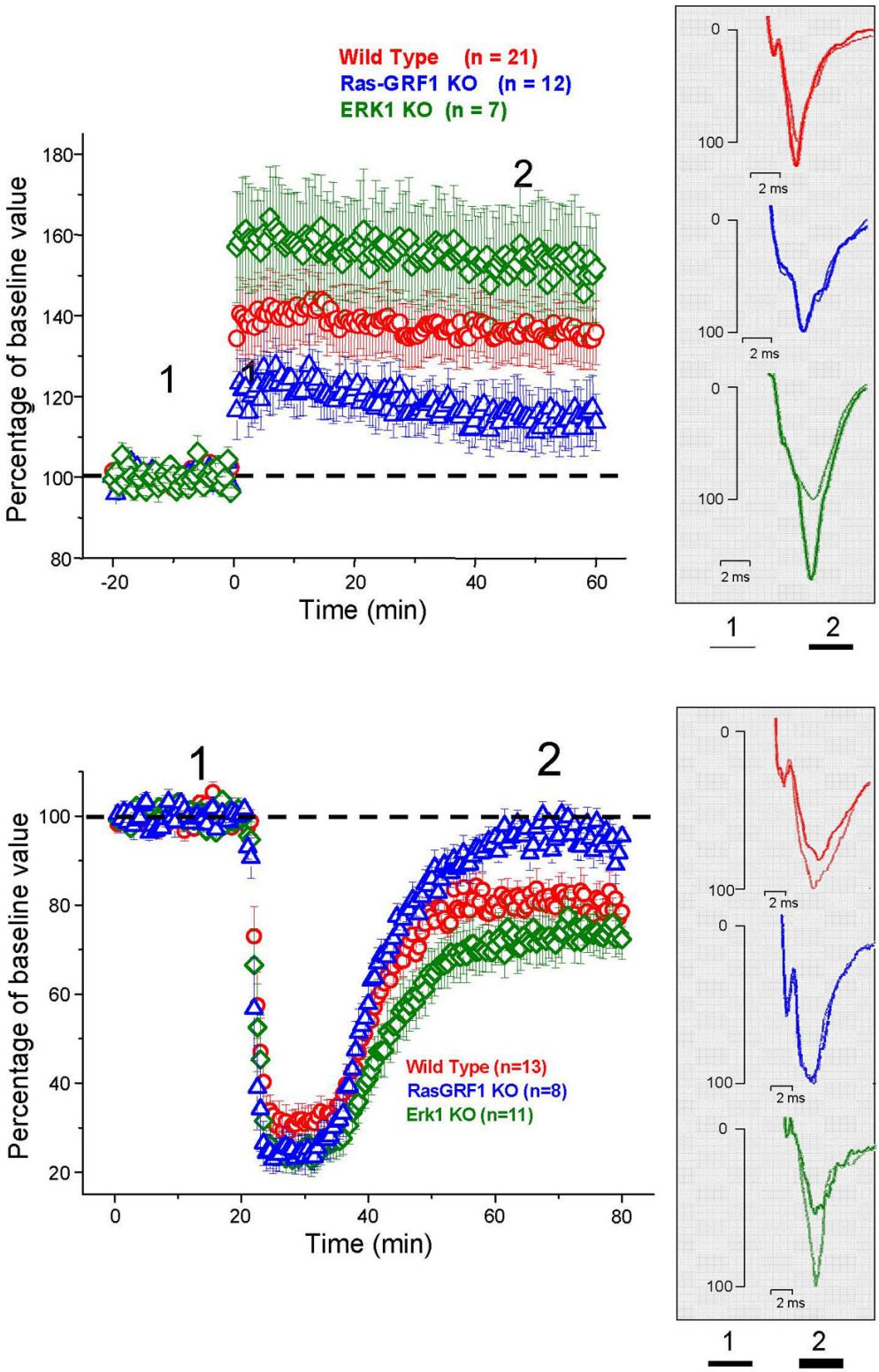

FIGURE 5 | Top: Average time course of field EPSP amplitude recorded from PRHC. The final level attained for LTP is significantly lower in Ras-GRF1 KO mice while it is higher in ERK1 KO mice with respect to WT mice (ANOVA, genotype × time, genotype $p < 0.001$, *post hoc* Student–Newman–Keuls Method). Bottom: Average time course of field EPSP amplitude recorded from PRHC. The final level attained for LTD in Ras-GRF1 KO mice is significantly lower than in WT mice; the latter is significantly lower than in ERK1 KO mice (ANOVA, genotype × time, genotype $p < 0.001$, *post hoc* Holm–Sidak method).

2003; Aggleton and Brown, 2005; Winters and Bussey, 2005; Murray et al., 2007; Griffiths et al., 2008; Massey et al., 2008; Winters et al., 2008).

RAS-GRF1 SIGNALING IS NECESSARY FOR RECOGNITION MEMORY CONSOLIDATION AND RECONSOLIDATION

ERK1 and ERK2 activation is the result of the integration of multiple signals deriving from neuronal activity and neurotrophin signaling, such as Ca^{2+} influx, activation of G-protein coupled receptors and trk receptor signaling (Weeber et al., 2002; Sweatt, 2004). Central to ERK activation is the increase in the active, GTP bound form of the small G-Protein Ras, which causes MEK phosphorylation. Ras activation, in its turn, is under control of guanine exchange factors, GEF, which catalyze the exchange of GDP for GTP on Ras, such as the Sos and the Ras-GRF families. Ras-GRFs activate Ras in response to Ca^{2+} signaling and muscarinic receptor activation (Farnsworth et al., 1995; Mattingly and Macara, 1996). Brambilla et al. (1997) have shown that mice with genetic deletion of Ras-GRF1, a guanine exchange factor which catalyzes the exchange of GDP for GTP on Ras (Ras-GRF1 KO mice) exhibit a reduced activation of ERK by neuronal activity and show deficits in amygdala dependent tasks such as active avoidance, inhibitory avoidance, and cued fear conditioning and also in contextual fear conditioning, which depends both on the amygdala and hippocampus. Giese et al. (2001), in a different mouse line with Ras-GRF1 inactivation (Ras-GRF1 deficient mice), found a clear deficit in spatial memory, which is hippocampus dependent. Nothing was known on the specific role of Ras-GRF1 signaling in visual recognition memory. Our results in Ras-GRF1 KO mice show that these mice have normal short term memory, as tested at 1 h delay from the learning session, but display a long term memory deficit; indeed, their performance differs from that of WT mice at the 12-h interval. This is indicative of a consolidation deficit. Thus the activation of ERK in the PRHC, which our data suggest to be necessary for the consolidation of long term recognition memory, has a crucial component in the Ras-GRF1 signaling.

Since our KO mice are not conditional, we cannot exclude developmental effects, although gross alterations have never been described in these mice (Brambilla et al., 1997; Mazzucchelli et al., 2002) and a recent paper (Tian and Feig, 2005) shows that Ras-GRF signaling is not involved in activating the Ras/ERK pathway in pubescent mice but only in adult mice. However, in absence of an acute interference/rescue of RAS-GRF1 or of ERK1 KO we cannot completely exclude developmental effects.

The consolidation deficit in Ras-GRF1 mice could be rescued by extended training. This suggests that it is possible to compensate for the lack of Ras-GRF1 mediated activation of the Ras-ERK pathway by Ras-GRF2 mediated activation of Ras-ERK (Li et al., 2006), thus preserving the action of neural activity on the Ras-ERK pathway; alternatively, or in addition, in mice subjected to repeated training other ERK activating pathways could be recruited, either impinging on Ras, such as the tyrosine kinase receptors pathways, or independent from Ras, such as the PKA pathway (Weeber et al., 2002; Sweatt, 2004).

It is important to note that also the process of reconsolidation is impaired in Ras-GRF1 KO mice, as shown by the lack of preferential exploration of the novel object 24 h after the reactivation of the memory trace in mice subjected to extended training, which, in absence of the intervening reactivation, would be able to recognize the familiar object up to 48 h after learning. ERK activation was known to be crucial both for consolidation and reconsolidation of recognition memory (Kelly et al., 2003); we now show that ERK activation needs the Ras-GRF1 mediated component to allow learned visual recognition memories to be consolidated and, if recalled, reconsolidated. It has been suggested that consolidation and reconsolidation might involve the activation of both shared pathways and specific pathways: indeed, Lee et al. (2004) found that BDNF and zif 268 were independently required in the hippocampus for consolidation and reconsolidation of contextual fear conditioning. Ras-GRF1 is evidently one of those molecules crucial for both processes, at least as far as recognition memory is concerned.

LONG TERM SYNAPTIC PLASTICITY DEFICITS IN THE PRHC OF RAS-GRF1 KO MICE

In very good correlation with the behavioral deficits, long term synaptic plasticity in the PRHC of Ras-GRF1 KO mice was defective. This lends further support to the role of PRHC plasticity in visual recognition memory (Warburton et al., 2003; Griffiths et al., 2008; Massey et al., 2008) and shows for the first time that ERK activation by Ras-GRF1 is crucial both for the LTD and the LTP type synaptic plasticity in the PRHC.

The LTD induction protocol we employed is dependent on cholinergic activity and it is known that Ras-GRF1 mediates the effects of muscarinic receptor activation (Farnsworth et al., 1995; Mattingly and Macara, 1996). The deleterious effects of Ras-GRF1 deletion on perirhinal LTD and on familiarity-based visual recognition memory are in good agreement with the crucial role of the cholinergic input to the PRHC via muscarinic receptors in this type of memory and in synaptic plasticity of the LTD type in the PRHC (Massey et al., 2001; Warburton et al., 2003).

In addition to the impairment in PRHC LTD type plasticity Ras-GRF1 KO mice also exhibit a deficit in perirhinal LTP. The combined effect on LTP and LTD we have found in Ras-GRF1 KO is reminiscent of the effects of NMDAR block shown by Barker et al. (2006): infusion of the NMDA receptor antagonist AP5 in the PRHC impairs recognition memory and both LTP and LTD. This is consistent with the role of Ras-GRF in mediating the effects of neuronal activity and in particular of Ca^{++} calcium signaling. Ras-GRF1 function has been indeed linked to Ca^{++} inflow through NMDA receptors (Li et al., 2006); interestingly, in the Barker et al. (2006) paper, blocking separately NMDA2A or 2B had effects on only one type of plasticity (LTP for the 2A block and LTD for the 2B block) and did not significantly impair recognition memory at the 24-h delay.

Ras-GRF1 could also mediate the effects of Ca^{++} influx via voltage dependent calcium channels (Farnsworth et al., 1995; Brambilla et al., 1997; Li et al., 2006), which, in a very recent study (Seoane et al., 2009), have been implied in recognition memory: block of a class of voltage dependent Ca^{++} channels in the PRHC impaired familiarity-based recognition memory and disrupted perirhinal LTD.

The importance of the Ras-GRF1 mediated ERK activation in PRHC plasticity both of the LTP and the LTD type is therefore

likely to rest on the integrative action Ras-GRF1 exerts with respect to intracellular signaling in perirhinal neurons. Thus, reduction of ERK activation by neural activity (Fasano et al., 2009) via Ras-GRF1 is sufficient to disrupt both the process of consolidation and reconsolidation of visual recognition memory, and PRHC synaptic plasticity both of the LTP and the LTD type.

INCREASED ERK2 ACTIVATION ENHANCES PERFORMANCE IN RECOGNITION MEMORY AND SYNAPTIC PLASTICITY IN PRHC

ERK1 KO mice might be considered an *in vivo* model of an enhanced susceptibility of ERK activation by the many pathways converging on MEK. ERK1 KO mice exhibit a stronger ERK2 activation (Mazzucchelli et al., 2002 and our results); this is explained in terms of the enhanced ERK2 phosphorylation by MEK due to the absence of the competing presence of ERK1. We have shown that this enhanced ERK2 activation is paralleled by an enhanced performance in visual recognition memory and by an enhanced synaptic plasticity in the PRHC. This is in accordance with Mazzucchelli et al. (2002), who found a superior performance of ERK1 KO with respect to wt mice in striatal dependent memory tasks (passive and active avoidance) and an enhanced striatal LTP. Thus, just as reducing ERK activation impairs visual recognition memory and synaptic plasticity in the PRHC, enhancing ERK activation produces the opposite effects.

ERK 1 and ERK 2 have been recently suggested to play different roles in intracellular signaling; in particular, it has been suggested that it is ERK2 which play the major role in ERK signaling (Mazzucchelli et al., 2002; Fremin et al., 2007; Bessard et al., 2008; Samuels et al., 2008). Our data confirm the importance of ERK2 signaling in synaptic plasticity and memory processes. We cannot exclude that in ERK1 KO mice there is a change in other pathways that contributes to recognition memory enhancement. However, intrahippocampal infusion of U0126 prevented contextual fear extinction in ERK1 KOs and their wild-type littermates (Tronson et al., 2008). It appears, therefore, that overactivation of the hippocampal ERK2 isoform by MEK predominantly contributed to the enhanced fear extinction phenotype of ERK1 KOs. In addition, in another recent paper in ERK1 KO mice, it has been shown (Alter et al., 2010) that systemic MEK inhibition with SL327 attenuated pain induced spontaneous behaviors similarly in wild-type and ERK1 KO mice, indicating that unrelated signaling pathways do not functionally compensate for the loss of ERK1.

We are aware that the relation between potentiating a signaling pathway and enhancing a memory process is not straightforward and may depend upon the type of memory or the type and site of intervention on the molecular cascade. For instance, mutation in NF1 (Costa et al., 2001, 2002) causes a constitutive hyperactivation of the Ras pathway which does not produce positive effects on memory. It has to be underlined that the Ras hyperactivation resulting from lack of a GAP protein (NF1) is, in fact, a disregulation of the Ras-ERK pathway and, in terms of ERK activation, it is very much different from the enhanced ERK2 phosphorylation caused by the physiological activation of MEK.

In conclusion, our results contribute to unravel the molecular basis of visual recognition memory, suggesting a pivotal role for Ras-GRF1 activation of ERK in the PRHC in both consolidation and reconsolidation processes and showing that modifying the gain in the ERK activation pathway bidirectionally affects visual recognition memory and synaptic plasticity in the PRHC, producing non-only an impairment, when the gain is reduced, but also an enhancement when the gain is increased.

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Molecular mechanisms underlying memory consolidation of taste information in the cortex

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The senses of taste and odor are both chemical senses. However, whereas an organism can detect an odor at a relatively long distance from its source, taste serves as the ultimate proximate gatekeeper of food intake: it helps in avoiding poisons and consuming beneficial substances. The automatic reaction to a given taste has been developed during evolution and is well adapted to conditions that may occur with high probability during the lifetime of an organism. However, in addition to this automatic reaction, animals can learn and remember tastes, together with their positive or negative values, with high precision and in light of minimal experience. This ability of mammalians to learn and remember tastes has been studied extensively in rodents through application of reasonably simple and well defined behavioral paradigms. The learning process follows a temporal continuum similar to those of other memories: acquisition, consolidation, retrieval, relearning, and reconsolidation. Moreover, inhibiting protein synthesis in the gustatory cortex (GC) specifically affects the consolidation phase of taste memory, i.e., the transformation of short- to long-term memory, in keeping with the general biochemical definition of memory consolidation. This review aims to present a general background of taste learning, and to focus on recent findings regarding the molecular mechanisms underlying taste–memory consolidation in the GC. Specifically, the roles of neurotransmitters, neuromodulators, immediate early genes, and translation regulation are addressed.

Keywords: taste learning, consolidation, gustatory cortex, insular cortex, MAPK, ERK, translation regulation, conditioned taste aversion

INTRODUCTION

Intake of food and avoidance of poison are crucial to an organism's survival in a dynamic environment. The process of deciding whether food is "safe" or "hazardous" relies heavily on the gustatory and somatosensory systems, which are involved in evaluating diverse properties of putative foods, such as: chemosensory, e.g., modality, intensity; orosensory, e.g., texture, temperature, pungency; and gratification capacity (Rosenblum, 2008; Carleton et al., 2010).

The sense of taste differs from the other senses in two major characteristics: (1) Each taste input has dual labeling, one related to its physical (texture and temperature) and chemical features, and the other to its hedonic value and safety; (2) Deciding on the safety aspects of a novel sensation, such as the taste of an unfamiliar food, involves a different temporal scale from other senses: taste association with unconditioned stimulus (US) takes 1–12 h (Bures et al., 1998; Merhav and Rosenblum, 2008), compared with a few seconds for responses to other sensations.

Two main strategies are used by various species, including humans, in responding to various tastes: genetic programming, i.e., affinity to sweet tastes, aversion to bitter ones, and complex learning mechanisms that involve several forebrain structures (Rosenblum, 2008). Modification of the basic genetic programming and memories of new tastes and their values are expected to be mediated, at least in part, by the gustatory cortex (GC; Yamamoto et al., 1984, 1985; Rosenblum, 2008; Doron and Rosenblum, 2010), which is defined according to its cytoarchitectonic boundaries as the dysgranular part of the insular cortex (IC; Burwell, 2001). This is in line with its unique anatomical connections (**Figure 1**), through which it receives multimodal sensory inputs, including visceral, gustatory, and somatosensory information from sensory thalamic nuclei (Fujita et al., 2010). However, other brain regions, such as the basolateral amygdala (Bla) and dorsal hippocampus, have been shown to be activated during novel taste processing (Yefet et al., 2006; Doron and Rosenblum, 2010), and also to be necessary for safe taste–memory consolidation (De la Cruz et al., 2008). Most of the studies of the molecular mechanisms underlying taste memory were performed in mice and rats, therefore, the present review will focus on these studies.

Taste recognition on the receptor level, as well as taste reactivity, as defined genetically, are beyond the scope of this review; they have been extensively covered elsewhere (Frank et al., 2008; Carleton et al., 2010). The first objective of this review is to describe taste-related behavior paradigms, in order to elucidate the molecular and cellular mechanisms underlying learning and memory. We then provide a detailed analysis of the currently known molecular and cellular mechanisms of taste learning in the GC, which resides in the IC. We aim to focus mainly on studies during the last decade; previous studies have been reviewed elsewhere (Bures et al., 1998; Rosenblum, 2008).

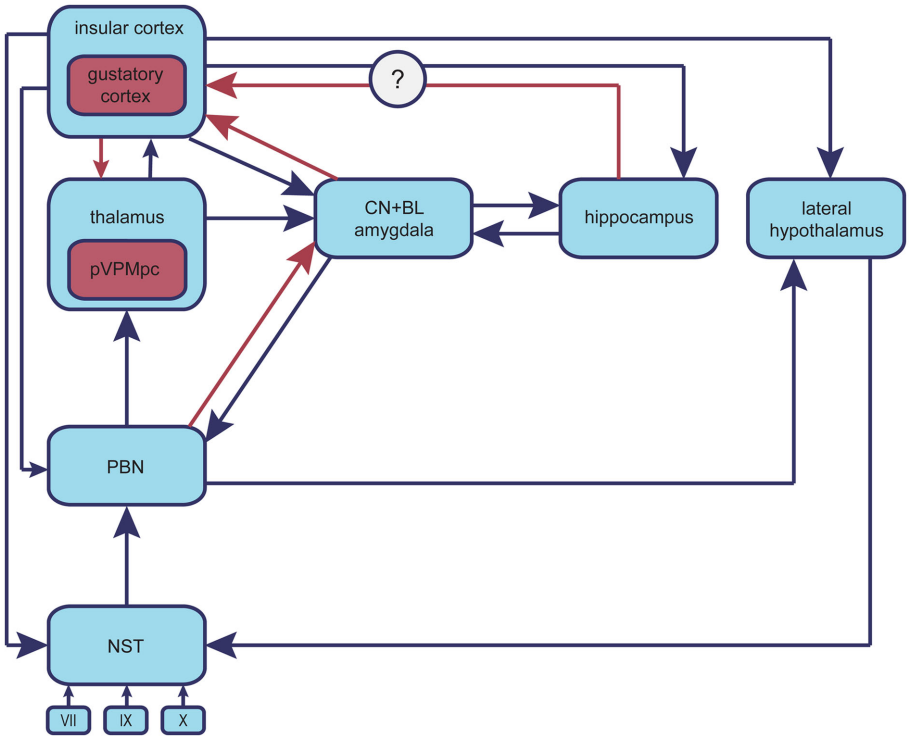

FIGURE 1 | Neuroanatomy of the taste system. Chemical stimuli originating in alimentary sources, upon reaching the oral cavity initiate the processing of gustatory information (CN, central nucleus; BLA, basolateral amygdala; NST, nucleus of solitary tract; PBN, parabrachial nucleus; pVPMpc, parvocellular part of the ventralis postmedial thalamic nucleus of the thalamus; GC, gustatory cortex). Taste cells, which are broadly tuned to the diverse taste modalities, are innervated by cranial nerves VII, IX, X, which project to the primary gustatory nucleus in the brainstem (NST). The NST sends information to three different systems: the reflex system, the lemniscal system, and the visceral–limbic system. The reflex system comprises medullary and reticular-formation neurons, which innervate the cranial motor nuclei. The lemniscal system consists of projections of the gustatory portion of the NST to the secondary nucleus situated in the dorsal pons (PBN); this, in turn, sends axons to the pVPMpc, which ultimately relays gustatory information to the anterior part of the insular cortex (GC). The visceral–limbic system refers to a collateral network of connections to the hypothalamus and limbic areas in the forebrain, which comprises the central gustatory pathway. The PBN is connected to the amygdala, the hypothalamus, and the bed-nucleus of the stria terminalis. All limbic gustatory targets are interconnected with each other as well as with the PBN and the GC.

BEHAVIOR PARADIGMS FOR THE MEASUREMENT OF TASTE LEARNING, MEMORY, AND CONSOLIDATION

The molecular mechanisms underlying learning and memory are the subject of ongoing research. Although learning and memory are considered to be two different processes, they involve a continuum of events. Following a discrete event, physical changes underlying memory encoding and processing of the information to be stored takes place. Ethologically, this is described as the acquisition phase; it involves creation of an internal representation of the novel information. This representation remains labile for some time, while the process of consolidation takes place. During this process the new memory becomes increasingly resistant to disruption (Alberini, 2011), which can involve several types of intervention: behavioral (e.g., Merhav et al., 2006), pharmacological (e.g., Rosenblum et al., 1993), or others (Bures et al., 1998). It has been shown that the consolidation of new memory can be disrupted by many events, including: blocking synthesis of new RNA or protein, e.g., by actimomycin D or anisomycin, respectively; disruption of the expression or function of specific proteins; new learning; brain cooling; seizure, e.g., through electric shock; brain trauma; and regional brain lesions (Alberini, 2011). During the consolidation phase, the memory is transformed from short-term memory (STM), which may last from minutes to hours, to long-term memory (LTM), which may last from days to a lifetime (McGaugh, 2000; Kandel, 2001; Dudai, 2004). The time frame of the "consolidation phase" can vary within a given learning paradigm; it depends on the manipulation, and may reflect several different cellular and molecular processes (**Figure 2**). One may ask whether the difference between consolidation and maintenance of memories is an artificial one, since the processes can also be regarded as continuous processing of the information by varied molecular and cellular mechanisms within a certain cortical area. They can also be considered in terms of temporally differential involvement of several brain structures.

The next phase of memory processing is use or retrieval of the memory, during which it is susceptible to further changes, associated with a process of reconsolidation, which mediates its restabilization. The stability of a memory depends on its age: new memories are sensitive to post-reactivation disruption, but older ones are more resistant (Alberini, 2011).

Ethologically, learning is usually classified into non-associative (habituation and sensitization), associative (relationships between

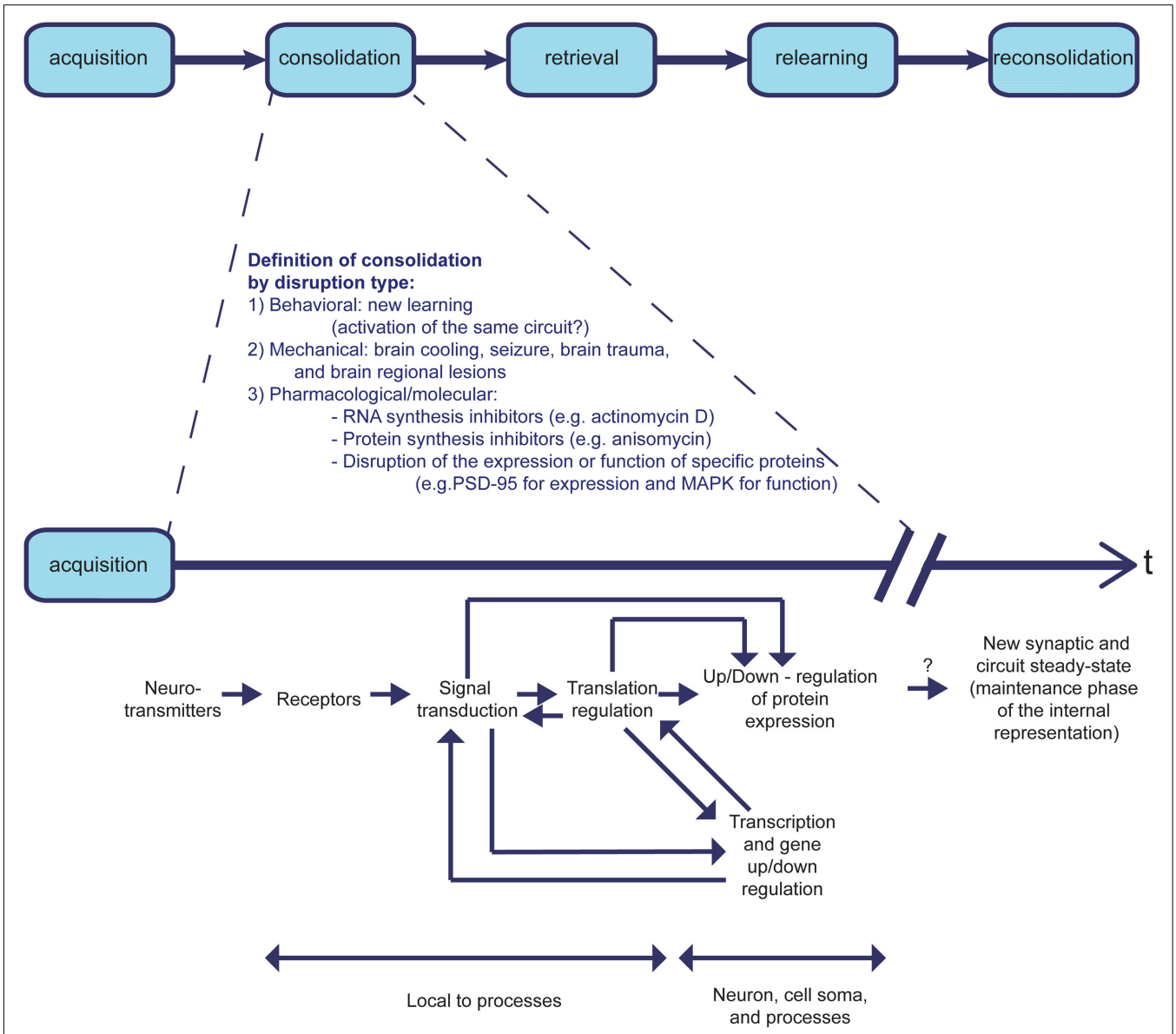

FIGURE 2 | The learning process phases: consolidation is determined by type of disruption. The learning process is ethologically divided into consecutive phases of acquisition, consolidation, retrieval, relearning, and reconsolidation. Whereas acquisition, retrieval, and relearning are defined in positive terms, the consolidation and reconsolidation phases are defined in negative terms. Therefore, the duration of the learning process is variable, and dependent on the type of experimental perturbation used to determine consolidation time. The different experimental interferences with consolidation affect different stages of the molecular mechanisms that underlie learning and memory formation. Although the same types of interference may be used to disrupt consolidation and reconsolidation, the time windows of sensitivity to disruption differ between the two phases of learning. It is therefore unclear whether the molecular processes that occur during consolidation are identical to the ones that occur during reconsolidation.

amounts and events), and incidental learning (learning in the absence of explicit external reinforcement; Gibb et al., 2006; Morris, 2006; Miranda et al., 2008; Rosenblum, 2008; Lindquist et al., 2009). Over the years, different behavioral paradigms have been developed in order to study the different types of learning, and also to address the abovementioned temporal phases of memory formation.

One of the most widespread taste-learning paradigms is the negative-learning, conditioned taste aversion (CTA) paradigm, in which an association is formed between a novel taste (serving as a conditioned stimulus – CS) and malaise (serving as an unconditioned stimulus – US), resulting in the animal's subsequent avoidance of the novel food associated with delayed food poisoning (conditioned response – CR; Garcia et al., 1955; Bures et al., 1998). The acquisition of CTA is subserved by specific brain regions, including the IC and the amygdala, although their precise role in CTA is still unclear (Yamamoto et al., 1994; Lamprecht and Dudai, 1996), and it has been demonstrated that induction of neurotoxic

lesions in the IC disrupts acquisition of CTA (Roman et al., 2006; Roman and Reilly, 2007). Similarly, lesions of the basolateral region (Bla), but not of the central nucleus of the amygdala, selectively disrupt CTA performance (Nachman and Ashe, 1974; Morris et al., 1999; St Andre and Reilly, 2007). CTA is commonly used when a hippocampus-independent form of learning is addressed.

A widely used paradigm of positive-learning is latent inhibition of CTA (LI-CTA), in which an animal learns that a novel food is safe, and displays less aversion following CTA than animals subjected to CTA alone (Bures et al., 1998). The LI-CTA paradigm involves presentation of a novel taste to an animal prior to CTA. Since this early experience elicits no negative consequences, the animal displays reduced aversion to the same taste following CTA (Rosenblum et al., 1993). This modulation of behavior can be attributed either to reduced strength of the association at the time of the CTA or to competition during the retrieval phase (Lubow, 1989). LI-CTA is a form of incidental learning that depends on both the quantity of novel taste consumed and the functionality of the GC (Rosenblum et al., 1993; Merhav and Rosenblum, 2008).

Under certain conditions, CTA can be extinguished (Berman et al., 2003). Extinction reflects a decrease in the CR in the absence of reinforcement of a conditioned stimulus. Behavioral evidence indicates that extinction involves an inhibitory learning mechanism in which the extinguished CR reappears following presentation of an unconditioned stimulus. However, studies have shown that the memory was not erased in rats and humans (Lin et al., 2010).

Taste learning and CTA have several advantages as paradigms for studying molecular mechanisms underlying learning and memory. These include: one-trial learning; strong incidental learning; clear and short learning time; minimal behavioral manipulation, since the animals can learn in their home cage with very little interference from other modalities; the sensory input is clearly defined, and therefore can be quantified in molecular terms; clearly defined cortical area(s) subserve the learning; and high reproducibility (Bures et al., 1998; Rosenblum, 2008).

LONG-TERM POTENTIATION IN THE INSULAR CORTEX

The most studied form of neuronal adaptations is Hebbian plasticity, which includes long-term potentiation (LTP), and its reciprocal, long-term depression (LTD; Collingridge et al., 2004; Malenka and Bear, 2004; Feldman, 2009; Pozo and Goda, 2010). Hallmark features of LTP are that synaptic changes are associative, rapidly induced, and input specific. These features facilitate reinforcement of active synaptic connections with a given set of sensory stimuli, thus eliciting an activity-induced increase in synaptic efficacy, which is widely expressed in several pleo- and neocortical areas. Taken together, these characteristics render LTP an attractive model for a cellular basis for learning and memory (Bliss and Collingridge, 1993; Neves et al., 2008; Rosenblum, 2008; Sjostrom et al., 2008).

Long-term potentiation has been described in several brain areas, including the hippocampus and the cortex, and in conjunction with taste learning, (Escobar et al., 1998, 2002; Ramirez-Lugo et al., 2003; Chen et al., 2006; Alme et al., 2007; Bramham, 2007; McGeachie et al., 2011; Rodriguez-Duran et al., 2011). It was demonstrated that tetanic stimulation of the basolateral amygdaloid nucleus (Bla) induced *N*-methyl-D-aspartate (NMDA)-dependent but metabotropic glutamate receptor (mGluR)-independent LTP in the IC (Escobar et al., 1998, 2002; Jones et al., 1999). It is important to note that IC–LTP and CTA both involve similar molecular mechanisms in the IC, such as NMDA receptor (NMDAR) dependence, activation of ERK1/2, and induction of immediate early genes (IEGs), including Zif268, Fos, Arc, and Homer (Jones et al., 1999; Rodriguez-Duran et al., 2011).

However, it remains to be more robustly proven that LTP-like mechanisms in the IC subserve taste learning. It was shown that induction of LTP in the Bla–IC projection prior to CTA enhanced CTA retention (Escobar and Bermudez-Rattoni, 2000). In addition, it was shown that on the one hand, intracortical microinfusion of brain-derived neurotrophic factor (BDNF) induced LTP in the Bla–IC projection of adult rats (Escobar et al., 2003), and on the other hand, that intracortical microinfusion of BDNF prior to CTA training enhanced retention of this task (Castillo et al., 2006).

In a follow-up study, Rodriguez-Duran et al. (2011) have shown that when the paradigm was reversed, i.e., CTA was performed prior to LTP induction in the Bla–IC projection, CTA training prevented the subsequent induction of LTP in the Bla–IC projection for at least 120 h after CTA training. In addition, they showed that CTA training produced a persistent change in the possibility of inducing subsequent LTP in the Bla–IC projection in a protein-synthesis-dependent manner, and inferred that changes in the possibility of inducing subsequent synaptic plasticity contributed to the formation and persistence of aversive memories (Rodriguez-Duran et al., 2011).

Long-term potentiation consists of two phases on the molecular level: induction, which triggers potentiation, and maintenance, which sustains the potentiation over time. Many molecules have been shown to be involved in the induction phase of LTP, but very few have been implicated in the maintenance phase. Since the working hypothesis is that LTP is the cellular mechanism underlying LTM storage, the molecular mechanisms relevant to the maintenance phase are highly important. To the best of our knowledge, to date, only a single molecule, protein kinase Mζ (PKMζ), has been found to be necessary for maintenance in both the hippocampus (spatial learning) and the IC (CTA): local injection of ZIP, a PKMζ selective inhibitor, into the hippocampus resulted in reversal of LTP maintenance *in vivo* and loss of 1 day old spatial memory (Pastalkova et al., 2006). Similarly, its injection into the GC resulted in reversal of long-term CTA memory in a dose-dependent manner, whereas other serine/threonine protein kinase inhibitors are capable of interference with long-term memory formation, but are ineffective once the memory has been established (Shema et al., 2009, 2011).

Protein kinase Mζ is the brain-specific atypical protein kinase C (PKC) isoform, which unlike full-length PKC isoforms, is a cleaved form comprising the independent catalytic domain of PKCζ, and is a second messenger-independent kinase. PKMζ is constitutively active in sustaining LTP maintenance, and studies have shown that it mediates synaptic potentiation specifically during the late phase of LTP. LTP induction increases new PKMζ synthesis, leading to enhanced synaptic transmission. The mechanism underlying L-LTP and spatial memory maintenance by PKMζ is thought

to involve AMPA receptor phosphorylation and trafficking, with subsequent changes in the amplitude of excitatory postsynaptic potential (EPSP) concomitant with dendritic translation regulated by PKMζ phosphorylation (and inhibition) of Pin1 (Sacktor, 2008; Vlachos et al., 2008; Navakkode et al., 2010; Parvez et al., 2010; von Kraus et al., 2010; Westmark et al., 2010; Mei et al., 2011; Sajikumar and Korte, 2011).

NEUROTRANSMITTERS IN THE GUSTATORY CORTEX INVOLVED IN TASTE LEARNING

The working hypothesis is that an organism relies on its sensory system, specifically, in the present context, the sense of taste, to create an internal representation of a given physical or chemical stimulus. The sensory information is converted into neuronal activity that subserves the various phases of learning, i.e., acquisition, consolidation, and retrieval. Information regarding physical/chemical properties and the significance of a given taste reaches the GC via several different neurotransmitters, and elicits the release of several neurotransmitter systems in the GC, where relevant receptors are expressed as well. Several molecular changes in the GC have been found to be correlated with novel taste learning at various time points after exposure to a novel taste. The molecules involved include acetylcholine (ACh), dopamine, noradrenaline, gamma-aminobutyric acid (GABA), glutamate, and various neuropeptides (**Figure 3**; Rosenblum et al., 1995, 1996, 1997; Berman et al., 2000; Belelovsky et al., 2005, 2009; Koh and Bernstein, 2005; Merhav et al., 2006; Banko et al., 2007; Costa-Mattioli et al., 2007; Elkobi et al., 2008; Merhav and Rosenblum, 2008; Barki-Harrington et al., 2009b; Doron and Rosenblum, 2010; Sweetat et al., 2011). However, only the muscarinic-cholinergic and NMDARs have been extensively studied with regard to their roles in taste–memory acquisition, consolidation, and retention (Jones et al., 1999; Gutierrez et al., 2003; Nunez-Jaramillo et al., 2008; Rosenblum, 2008).

GLUTAMATE

Physical and chemical taste information is transferred from the oral cavity to the cortex via fast neurotransmission, mediated by the neurotransmitter glutamate, the main excitatory neurotransmitter in the mammalian CNS (Rosenblum, 2008; Rondard et al., 2011). Since the prominence of a given taste is hypothesized to be mediated via activation of the neuromodulatory system (e.g., Kaphzan et al., 2006), it is possible that the interaction between the two systems produces a long-term taste–memory trace; it is also likely that it coincides on specific neurons and probably molecules that can serve as coincidence detectors of the sensory input and its meaning (Kaphzan et al., 2006).

The glutamate receptor family comprises four types of receptors: alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA), *N*-methyl-D-aspartic acid (NMDA), kainate, and mGluRs. AMPA, NMDA, and kainate receptors are ionotropic receptors, i.e., they can produce complex fast ion influx-mediated changes in the neuron, whereas mGluRs produce slow second messenger-mediated changes in the neuron by activating G-protein-coupled receptors (GPCRs; Rondard et al., 2011).

Glutamate and dopamine have been implicated in off-line processing and memory consolidation following CTA, by means of *in vivo* microdialysis and capillary electrophoresis (Guzman-Ramos et al., 2010). In their study, Guzman-Ramos et al. (2010) demonstrated the occurrence of an amygdala-dependent dopamine and glutamate reactivation within the IC, about 45 min after the stimuli association. Furthermore, blockade of dopaminergic D1 and/or NMDARs before the off-line activity impaired long- but not STM, which suggests dependence on protein synthesis (Guzman-Ramos et al., 2010). In addition, dopamine and NMDA can synergistically activate extracellular signal-regulated kinase (ERK)/Mitogen-activated protein kinase (MAPK) signaling, which is necessary for the formation of long-term taste memory (Kaphzan et al., 2006, 2007).

Activation of NMDARs has been shown to be necessary for attenuation of the neophobic taste response in both the IC (Figueroa-Guzman et al., 2006) and the Bla (Figueroa-Guzman and Reilly, 2008). Acute microinfusions of MK-801, a non-competitive NMDAR antagonist, into both brain regions revealed that although there was no effect on the initial magnitude of the neophobic response, attenuation of gustatory neophobia was prevented. Similarly, microinfusion of mGlu5-selective antagonist, 3-[2-methyl-1,3-thiazol-4yl)ethynyl]pyridine (MTEP), at 0, 1, or 5 μg into the rat IC or Bla prior to CTA resulted in enhanced CTA performance in the case of the Bla, indicated by robust CTA followed by slower extinction than in control animals, in a dose-dependent manner. Interestingly, MTEP microinfusion into the IC resulted in less robust aversion following CTA, in addition to enhanced extinction, in a dose-dependent manner (Simonyi et al., 2009). Previous studies that employed systemic administration of mGlu antagonists before CTA conditioning have demonstrated that activation of mGlu5, but not of mGlu1 receptors, was required for CTA learning (Schachtman et al., 2003). In addition, attenuation of CTA can be attained by microinjection of a broad-spectrum mGlu antagonist into the Bla (Yasoshima et al., 2000) or the IC (Berman et al., 2000).

mGlu5 receptors interact with NMDARs, and the two modulate one another's function in several brain regions in a mutually positive manner: stimulation of either receptor potentiates the other (Fowler et al., 2011). The two receptors are physically connected with each other through anchor proteins: mGlu5 receptor binds Homer proteins (Fagni et al., 2004), NMDAR interacts with postsynaptic density (PSD)-95, and Homer and PSD-95 can be clustered by Shank – all three of which are PSD proteins (Naisbitt et al., 1999; Tu et al., 1999). NMDA and mGlu5 can act synergistically to activate a number of proteins such as MAPKs, calcium/calmodulin-dependent protein kinase II (CaMKII), and CREB (Mao and Wang, 2002; Yang et al., 2004).

A plethora of studies using genetic, pharmacological, physiological, and biochemical approaches have indicated the critical roles played by mGlu5 and NMDA in both the IC and the hippocampus in acquisition and consolidation, but not retrieval, of memory of aversive tasks, specifically in avoidance learning and CTA (Schachtman et al., 2003; Cui et al., 2005; Gravius et al., 2005; Izquierdo et al., 2006; Simonyi et al., 2007, 2009; Barki-Harrington et al., 2009a; Nunez-Jaramillo et al., 2010). In a recent follow-up study employing co-administration of an mGlu5-receptor-positive allosteric modulator, 3-cyano-*N*-(1,3-diphenyl-1*H*-pyrazol-5-yl) benzamide (CDPPB), and an NMDAR

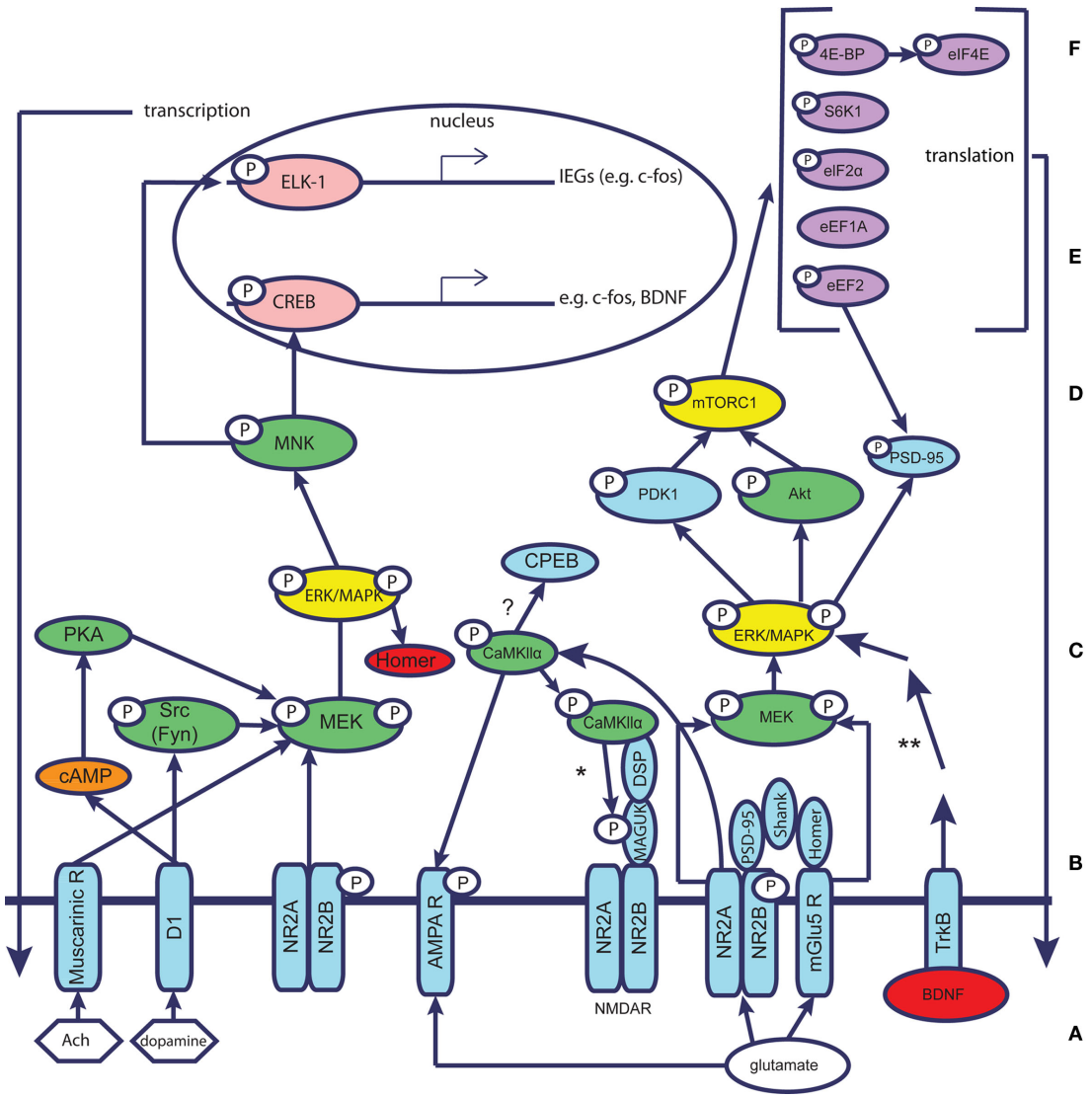

FIGURE 3 | Molecular mechanisms underlying taste learning in the gustatory cortex: schematic simplified representation assuming the different molecular events take place within the same neuron. Glutamate/neuromodulators (Ach, dopamine) reach the postsynaptic site and affect the neuron through specific receptors (NMDAR, AMPAR, mGluR, muscarinic receptors, dopamine receptors, e.g., D1). The receptors are linked to scaffold proteins that form the postsynaptic density (PSD), e.g., MAGUKs, Shank, Homer. MAGUKs are linked through distal scaffolding proteins (DSP) to CamKII. The complexes of receptors and PSD proteins activate signal transduction and hub molecules, which in turn activate transcription and translation regulation. This chain of events, according to our current understanding, results in a new cellular steady state. It is currently unknown whether all molecular processes depicted occur within a single neuron – a question that remains to be further explored. A, neuromodulators/glutamate; B, receptors; C, scaffold proteins; D, signal transduction and hub molecules; E, translation regulation; F, transcription. Key: neuromodulators – white hexagon; glutamate – white ellipse; receptors of neurotransmitters/neuromodulators – blue; representatives of major protein kinases and phosphatases that may have short-term (CamKIIα) or long-term effects-green; protein translation machinery – purple; transcription factors – coral; immediate early genes – red; second messenger – orange. *CaMKII is known to phosphorylate PSD-95 and SAP-97, but it remains to be clarified whether it phosphorylates PSD-93 and SAP-102 as well. In addition, CamKII is well known to activate cytoplasmic polyadenylation element binding protein (CPEB) in other brain regions and in connection with other forms of learning, thereby affecting protein translation. **The BDNF pathway has been simplified; other proteins participate in this pathway.

antagonist, MK-801, it was shown that NMDA and mGlu5 interacted functionally in CTA-conditioned rats. Whereas CDPPB administered by itself prior to the conditioning trial had no effect on CTA or hippocampus-dependent step-down inhibition, co-administration of the two compounds resulted in attenuation of learning deficits in both tasks (Fowler et al., 2011).

There is ample evidence in the literature for the importance of NMDAR, specifically its regulatory NR2B subunit, in taste learning. For example, CTA conditioning-induced long-lasting tyrosine phosphorylation of NR2B specifically in the IC (Rosenblum et al., 1995). Furthermore, local administration of (2*R*)-amino-5-phosphonovaleric acid (APV), a reversibly selective competitive

inhibitor of NMDAR, to the IC prior to CTA conditioning impaired CTA memory in a brain-region- and time-dependent manner (Rosenblum et al., 1995, 1996, 1997). In addition, whereas exposure to a novel taste, e.g., saccharin, increased phosphorylation in the NMDAR subunits, repeated doses of saccharin at the same concentration, which rendered the taste familiar, led to a dramatic decrease in the levels of serine phosphorylation of NR2A and NR2B subunits (Nunez-Jaramillo et al., 2008).

NR2B phosphorylation in the IC is not only correlated with but is necessary for taste learning, as has been demonstrated through pharmacological and genetic approaches: local administration of the tyrosine kinase inhibitor, genistein, to the IC prevented the increase in phosphorylation of NR2B on tyrosine 1472, and attenuated taste–memory formation (Barki-Harrington et al., 2009b). Additionally, whereas novel taste exposure has been recently demonstrated to induce intracellular redistribution of NR2A and NR2B subunits in the IC (Nunez-Jaramillo et al., 2008), microinjection of genistein to the IC altered this learning-induced distribution pattern of NMDAR, highlighting the importance of NR2B tyrosine phosphorylation after learning in determination of NMDAR distribution (Barki-Harrington et al., 2009b).

In another study, transgenic (Tg) mice over-expressing the NR2B subunit specifically in the forebrain (which includes the IC) were shown to have enhanced CTA, as well as slower extinction rates, although aversion levels were similar in Tg and wild-type (Wt) mice 30 days after the CTA conditioning. However, under the LI-CTA paradigm, the Tg mice did not differ from Wt mice in their aversion levels, and in a paradigm of two-taste LI-CTA (second order conditioning, in which the mice are exposed to both novel and familiar tastes) the Tg mice showed attenuation of enhanced CTA (Li et al., 2010).

GABA AND ACh

Although the involvement of glutamate receptors in the processing of taste learning in the GC has been extensively studied, other neurotransmitters have been implicated as well. Novel tastes have been shown to increase ACh levels in the rat IC, whereas a familiar taste did not (Miranda et al., 2000). Furthermore, pharmacological inactivation of the nucleus basalis magnocellularis, a cholinergic and GABAergic source in the basal forebrain, impaired CTA acquisition (Miranda and Bermudez-Rattoni, 1999). However, retrieval was not impaired by this manipulation. In addition, cholinergic activity mediated by muscarinic receptors in the IC has been shown to be necessary for acquisition and consolidation of contextual memory of inhibitory avoidance (Miranda and Bermudez-Rattoni, 2007).

Inhibitory GABAergic interneurons have been recently demonstrated to be activated in response to novel taste learning in a layer-specific manner hours after taste learning, which suggests that these neurons are involved not only in acquisition, but also in off-line processing and consolidation of taste information (Doron and Rosenblum, 2010). Electrophysiological studies in anesthetized rats revealed that excitatory propagation in the IC was primarily regulated by AMPA and $GABA_A$ receptors (Fujita et al., 2010). Another electrophysiological study in rat-cortex slices, which employed multiple-whole-cell patch-clamp recording from layer V GABAergic interneurons and pyramidal cells of rat IC, demonstrated that carbachol, a cholinergic agonist, increased the amplitude of unitary inhibitory postsynaptic currents (uIPSCs) in interneuron-to-interneuron synapses with higher paired-pulse ratios. However, the same compound induced dose-dependent suppression of uIPSCs in fast spiking of pyramidal cell synapses. This attenuation was mitigated by atropine, a muscarinic ACh receptor antagonist (Yamamoto et al., 2010), and the authors inferred that "carbachol facilitates GABA release in interneuron synapses with lower release probability, and cholinergic modulation of GABAergic synaptic transmission is differentially regulated depending on postsynaptic neuron subtypes." It is important to note that neurons projecting from one brain-region involved in taste learning to another (**Figure 1**) are inherently excitatory, however, neurons within a certain brain region may be inhibitory as well. The roles of inhibitory cortical neurons are yet to be determined.

POSTSYNAPTIC DENSITY-95

Postsynaptic density-95 (PSD-95), a membrane-associated guanylate kinase (MAGUK), is the major scaffolding protein in the excitatory PSD and a potent regulator of synaptic strength (Chen et al., 2011). It has been recently shown that 3 h following novel taste learning, expression levels of this protein were specifically elevated in the GC. This elevation has been shown to be necessary for acquisition of novel taste memory, but not for its retrieval, and it has not been observed in response to a familiar taste (Elkobi et al., 2008). Moreover, there was a correlative increase in PSD-95 association with tyrosine-phosphorylated NR2B following novel taste learning (Barki-Harrington et al., 2009b). A study concerning spatial learning, which is both hippocampus- and IC-dependent, has shown that water-maze training induced a translocation of NMDARs and PSD-95 to lipid raft membrane microdomains, concomitant with increased NR2B phosphorylation at tyrosine 1472 in the rat IC (Delint-Ramirez et al., 2008), similarly to novel taste learning, as mentioned above (Barki-Harrington et al., 2009b).

PSD-95 and other MAGUK family proteins, SAP-97 and PSD-93, have been shown to interact with NMDAR, thereby regulating its function, e.g., all three inhibited NR2B-mediated endocytosis (Lavezzari et al., 2003). The MAGUK family proteins have been shown to mediate NMDAR clustering and/or trafficking by association with NMDAR NR2 subunits via their C-terminal glutamate serine (aspartate/glutamate) valine motifs. In addition, the MAGUK proteins interacted differentially with different NMDAR subtypes, comprised of differing receptor subunit combination (Cousins et al., 2008). Specifically, PSD-95 interacted with both NR2A and NR2B (Kornau et al., 1995), and this interaction could be modulated by either serine or tyrosine phosphorylation.

For example, CaMKII phosphorylated PSD-95 on serine residue, causing dissociation of NR2A, but not of NR2B from the NMDA–PSD-95 complex. In addition, PSD-95 itself functioned as a negative regulator of the tyrosine kinase Src, for which it served as a contact point to the NMDAR, thus enabling its regulation (Kalia et al., 2006). Moreover, phosphorylation of NR2A and NR2B and their associated proteins by Src or Fyn was found in some cases to enhance their association with PSD-95 (Rong et al., 2001; Zalewska et al., 2005). Other tyrosine kinases shown

to interact with PSD-95 include c-Abl and Pyk2: the former regulated synaptic clustering of PSD-95 (de Arce et al., 2010); the latter underwent PSD-95-induced postsynaptic clustering and activation (Bartos et al., 2010). It has been suggested that the role of the PSD-95–NMDA complex is to protect NR2 subunits from undergoing cleavage by calcium-dependent proteases, and thereby to provide a mechanism for regulating NMDAR expression (Dong et al., 2004).

THE ROLE OF THE MAPK/ERK PATHWAY IN THE GUSTATORY CORTEX

Mitogen-activated protein kinases are a family of serine/threonine kinases implicated in regulation of cell proliferation and differentiation (Seger and Krebs, 1995; Belelovsky et al., 2007). Three major groups of MAPKs have been identified in mammalian cells: extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAP kinase. High levels of the ERK isoforms, ERK1 (p44 MAPK) and ERK2 (p42 MAPK), have been detected in neurons in the mature CNS (Fiore et al., 1993). ERK activity has been shown to be crucial for several forms of learning and memory, including fear conditioning, CTA memory, spatial memory, step-down inhibitory avoidance, and object recognition memory. In a study in which several pharmacological agents were locally injected into the rat IC, it was found that NMDARs, mGluRs, muscarinic, beta-adrenergic, and dopaminergic receptors were all implicated in acquisition of the new taste memory, but not in its retrieval, although these neurotransmitter/neuromodulator systems differed in their role in acquisition. In addition, it was demonstrated that of all the receptors studied, only NMDA and muscarinic receptors specifically mediated taste-dependent activation of ERK1–2, whereas dopaminergic receptors regulated the basal level of ERK1–2 activation (Berman et al., 2000). Several studies have demonstrated the differential role of ERK1 and 2 in cell growth and proliferation and synaptic plasticity. For example, ERK1 knockout mice displayed enhancement of striatum-dependent long-term memory, in conjunction with facilitation of LTP in the nucleus accumbens, indicating the importance of ERK2 (Mazzucchelli et al., 2002; Vantaggiato et al., 2006).

ERK activation occurs downstream to neurotransmitter release and activation of the forebrain cholinergic neurons during and immediately after acquisition of an inhibitory avoidance response. ERK plays a major role in learning, by promoting cellular integration of divergent downstream effectors that may trigger differing responses, depending upon which subsets of scaffolding anchors, target proteins, and regulatory phosphatases are involved (Giovannini, 2006). MEK–ERK, the upstream kinase of ERK, is a crucial signal transduction cascade in synaptic plasticity and memory consolidation (Sweatt, 2001), and its inhibition affected both early and late phases of LTP in the hippocampus (Rosenblum et al., 2002).

Extracellular signal-regulated kinase activation was shown to be correlated with novel taste learning, although the amount of protein remained unchanged (Berman et al., 1998; Belelovsky et al., 2005). In addition, the expression of LTP in the GC was ERK-dependent, and operated in a positive feedback mode (Jones et al., 1999). In a recent study, it was shown that bilateral injection of U0126, a specific MEK inhibitor, to the GC prior to learning resulted in inhibition of ERK1/2, as well as attenuation of CTA (Rosenblum, 2008) and blockade of learning-induced elevation of PSD-95 3 h following taste learning (Elkobi et al., 2008).

The various MAPKs are activated within differing time periods after novel taste learning; ERK activation appears to begin a few minutes up to 1 h following novel taste consumption (Rosenblum, 2008). It has been shown that novel taste consolidation requires the elevation of ERK1/2 activity in the IC 20 min after taste consumption. However, JNK1/2 was activated 1 h after novel taste learning, whereas p38 was not modified at any of the examined time points (Berman et al., 1998). Interestingly, the time scale of ERK activation was species-specific; it was shown to differ between mice and rats (Swank and Sweatt, 2001). ERK expression following taste learning is not only time-restricted, but also space-restricted; it was activated in the GC, but not in the hippocampus, after the same length of time following learning (Yefet et al., 2006). The various MAPKs also differed in BDNF-induced activation: intrahippocampal microinfusion of BDNF that aimed to induce LTP resulted in rapid phosphorylation of ERK and p38, but not of JNK (Ying et al., 2002). These effects were observed in the dentate gyrus, but not in other examined hippocampal regions, and were shown to be MEK–ERK-dependent.

Whereas the upstream regulation of ERK in the GC is well studied, our knowledge regarding its downstream targets remains fragmentary. MAPK substrate, ELK-1, a transcription factor regulating immediate early expression of genes via the serum response element (SRE) DNA consensus site (Besnard et al., 2011), has been shown to be phosphorylated in a time frame similar to that of ERK activation after novel taste learning, and neurotransmitters required for induction of LTM are also required for ELK-1 phosphorylation in the GC in response to taste learning (Berman et al., 2003). Further studies are needed to identify other possible targets of ERK and their mechanisms of action underlying taste processing in the GC.

THE ROLE OF TRANSLATION REGULATION IN TASTE–MEMORY CONSOLIDATION

The distinctive biochemical characteristic of memory consolidation is its dependence on synthesis of functional proteins in the relevant brain regions (Davis and Squire, 1984). Indeed, local application of anisomycin, a protein-synthesis inhibitor, to specific brain regions affected CTA in a dose-, site-, and time-dependent manner (Rosenblum et al., 1993; Meiri and Rosenblum, 1998). For instance, local application of anisomycin to the GC attenuated CTA and taste learning under the LI paradigm (Rosenblum et al., 1993); however, the same treatment had no effect on STM (Houpt and Berlin, 1999), which suggests that short-term taste memory is independent of protein synthesis. Temporally, taste learning is sensitive to protein-synthesis inhibitor(s) from just before learning until up to 100 min afterward (Rosenblum, 2008). In addition, extinction of CTA is dependent on functional protein synthesis in the prefrontal cortex (Akirav et al., 2006). It is generally accepted that protein translation affects LTM consolidation by modulation of synaptic strength, since protein-synthesis inhibitors prevented transformation of early LTP to late LTP. However, other modifications of intrinsic neuronal properties also subserve learning-related behavioral changes. For

example, learning-induced reduction in the postburst after hyperpolarization (AHP) lasts for days after completion of training, and is implicated in maintenance of learned skills. It has been recently demonstrated that synaptic activation-induced short-term postburst AHP reduction in hippocampal and pyramidal neurons could be transformed to a protein-synthesis-dependent long-term AHP reduction that persisted for long time periods (Cohen-Matsliah et al., 2010). Such learning-induced AHP reduction has been shown to be maintained by persistent activation of PKC and ERK in piriform cortex neurons (Seroussi et al., 2002; Cohen-Matsliah et al., 2007), but has been found to be CaMKII-independent (Liraz et al., 2009).

Translation consists of three phases: initiation, elongation, and termination. Of these, the initiation phase is the most tightly regulated, and is affected by phosphorylation of initiation factors (IFs) and ribosomal proteins (Proud, 2000). Initiation in eukaryotes serves as the rate-limiting step in protein synthesis, and therefore serves as an important target for translational control (Costa-Mattioli et al., 2009). There is considerable evidence in the literature that increased initiation results in enhanced learning, and vice versa. In knockout mice lacking the translation repressor eukaryotic initiation factor 4E-binding protein (4EBP2; Banko et al., 2007) or with reduced phosphorylation of eukaryotic initiation factor 2α (eIF2α; Costa-Mattioli et al., 2007), and therefore with enhanced initiation, no differences in taste recognition in parallel to enhanced CTA learning were observed; furthermore, other types of learning and plasticity in the consolidation phase were enhanced as well. Conversely, knockout mice lacking either S6K1 or S6K2, characterized by reduced initiation rates, exhibited impaired taste learning (Antion et al., 2008). In a model analogous to CTA in the chick, eukaryotic translation initiation factor 2B (eIF2B) was found to be both correlated with and necessary for taste–memory consolidation (Tirosh et al., 2007).

The second phase of translation, elongation, requires activity of elongation factors (EFs). Eukaryotic elongation factor 2 (eEF2) mediated ribosomal translocation (Ryazanov and Davydova, 1989) and was phosphorylated on Thr56 by a specific Ca^{2+}/calmodulin-dependent kinase. Phosphorylation of this kinase inhibited its activity and led to general inhibition of protein synthesis (Nairn and Palfrey, 1987). It has been shown that following novel taste learning, eEF2 phosphorylation was increased in the GC, indicating attenuation of translation elongation (Belelovsky et al., 2005). This finding, which is counter-intuitive, implies that the situation is more complex than implied by the simple model of "the more IFs, the better the taste learning" (Rosenblum, 2008). Nevertheless, there was increased initiation in the same samples, manifested as increased phosphorylation levels of ERK and S6K1 (Belelovsky et al., 2005).

Therefore, we have proposed a putative mechanism, by which increased initiation concomitant with decreased elongation in the same neurons in the GC might increase expression levels of mRNAs that are poorly initiated. This was reflected, for example, in the case of phosphorylation of the α subunit of eIF2 (eIF2 α), which, in turn, led to suppression of general translation (Hinnebusch et al., 2000), concomitant with stimulation of translation of ATF4 (Lu et al., 2004; Vattem and Wek, 2004), which is required for late phase LTP and LTM (Bartsch et al., 1995; Chen et al., 2003). The suggested mechanism could perform a switch-like function in expressing a specific set of mRNAs within a restricted time window in a cellular microdomain/s such as the synapse.

MAMMALIAN TARGET OF RAPAMYCIN

Some of the correlative changes that follow novel taste or CTA paradigms have been observed in proteins that are either direct or indirect targets of the mammalian target of rapamycin (mTOR), also known as FKBP-12-rapamycin-associated protein (FRAP), which consists of two TOR complexes (TORC) that differ in rapamycin sensitivity. TORC1 mediated rapamycin-sensitive TOR-shared signaling to the translation machinery, the transcription apparatus, and other targets (Loewith et al., 2002; Hay and Sonenberg, 2004), and its blockade by rapamycin interfered with translation of specific subpopulations of mRNA (Raught et al., 2001). Downstream targets of mTOR include ribosomal protein kinase (S6K1) and EF 1A and 2 (eEF1A and eEF2), which are mostly involved in ribosome recruitment to mRNA, and regulation of both the initiation and elongation phases of translation (Hay and Sonenberg, 2004).

There is considerable evidence for the importance of the mTOR pathway in various forms of synaptic plasticity. In *Aplysia*, rapamycin application prevented long-term facilitation (Casadio et al., 1999), and in the rat hippocampal CA1 region, rapamycin blocked high-frequency stimulation (HFS) and BDNF-induced LTP (Tang et al., 2002). In addition, mTOR-dependent activation of dendritic S6K1 was shown to be necessary for the induction phase of protein-synthesis-dependent synaptic plasticity (Cammalleri et al., 2003).

It has been recently shown that following novel taste learning, two temporal waves of mTOR activation occurred in the GC (Belelovsky et al., 2009). Furthermore, it was shown that PSD-95 elevation in the GC 3 h following taste learning, which is necessary for LTM (Elkobi et al., 2008), was prevented following local application of rapamycin to the GC. Another study has shown, by means of HFS, an interesting interplay between ERK and mTOR pathways induced at CA3–CA1 synapses: whereas HFS induced LTP as well as translational proteins regulated by mTOR, the former induction was blocked by use of ERK inhibitors (Tsokas et al., 2007). Moreover, this study showed ERK to be not only correlated with, but necessary for mTOR stimulation by HFS via interaction with phosphoinositide-dependent kinase 1 (PDK1) and Akt, which are upstream to mTOR.

Other studies that used genetic and pharmacological approaches in the CA1 region of the hippocampus showed that activation of mTORC1 facilitated initiation of protein translation through phosphorylation and inhibition of eukaryotic initiation factor 4E (eIF4E)-binding proteins (4E-BP), which inhibit complex formation. Phosphorylation of eIF4E on Ser209 is ERK-dependent and was closely linked with translation of specific mRNA subpopulations (Panja et al., 2009). Thus, it is currently accepted that ERK and mTORC1 synergistically regulate eIF4E and translation initiation in LTM and synaptic plasticity. It has been recently shown that Pin1 inhibited protein-synthesis induced by glutamatergic signaling, and it was suggested that eIF4E and 4E-BP1/2 mediated an increase in dendritic translation induced by Pin 1 inhibition (Westmark et al., 2010).

It is important to note that other mechanisms have been implicated in translational control of long-lasting synaptic plasticity and memory, e.g., micro-RNA regulation (miRNA), and it is highly likely that they participate, at least in part, in taste learning as well. However, it remains to be clarified whether this is indeed the case.

IMMEDIATE EARLY GENES AND CONVERGENCE OF CS/US

Immediate early genes, activated transiently and rapidly by various cellular stimuli, are regulated at the transcription level. They provide a first response to these stimuli, in advance of protein synthesis. Many of the IEGs are transcription factors or other DNA-binding proteins (Plath et al., 2006). Expression levels of IEGs have been shown to increase in neuronal populations that subserve stimulus encoding in response to various kinds of behaviors (Campeau et al., 1991; Guzowski et al., 2005; Mattson et al., 2008; Koya et al., 2009). Several such IEGs have been implicated in taste memory, including cFOS, Activity-regulated cytoskeleton-associated protein (Arc/Arg3.1), Homer, and BDNF (Saddoris et al., 2009; Doron and Rosenblum, 2010), and have been shown to be elevated following other forms of learning as well. The aforementioned IEGs belong to a subclass termed effector neuronal IEGs, which mediate NMDAR-regulated phenotypic changes in the brain (Kaufmann and Worley, 1999). Many of these have been immunohistochemically detected in dendrites (Lyford et al., 1995; Tsui et al., 1996).

ARC AND HOMER

Arc and Homer are NMDAR-dependent markers for plasticity (Guzowski et al., 2001). Arc has been shown to play an important role in consolidation of synaptic plasticity and memories as an effector molecule downstream of many signaling pathways (Shepherd and Bear, 2011). It mediates synaptic homeostatic scaling of AMPA receptors, via a mechanism of interaction with the endocytosis machinery which, in turn, regulates AMPA receptor trafficking (Chowdhury et al., 2006; Plath et al., 2006; Shepherd et al., 2006). Additionally, Arc affects cytoskeletal dynamics, as local injection of Arc antisense into the dentate gyrus 2 h following LTP induction resulted in reversal of LTP, as well as dephosphorylation of actin depolymerization factor/cofilin, and loss of nascent filamentous actin (F-actin) at synaptic sites (Messaoudi et al., 2007; Bramham et al., 2010). In turn, these changes are instrumental in regulation of spine morphology by increasing spine density (Peebles et al., 2010). A recent electrophysiological study has shown that Arc synthesis was regulated by ERK–MNK signaling during LTP consolidation in the dentate gyrus in live rats. Although mTORC1 is activated following HFS stimulation, its pharmacological inhibition revealed that it is not essential for Arc synthesis and LTP (Panja et al., 2009).

Homer1 is a PSD scaffolding protein, involved in the regulation of synaptic metabotropic receptor function; it has been implicated in structural changes occurring at synapses during long-lasting neuronal plasticity and development (Xiao et al., 1998). Both Arc and Zif268 are required for generation of mRNA-dependent LTP. Additionally, intrahippocampal microinfusion of BDNF resulted in selective upregulation of Arc mRNA and protein, in addition to rapid and extensive delivery of Arc mRNA transcripts to granule cell dendrites (Ying et al., 2002).

A recent study has elegantly exploited the fact that Arc and Homer1a show differential temporal expression patterns in activated neurons (Guzowski et al., 2001): it was used to mark neuronal ensembles in the GC and Bla that participated in processing novel taste information. Using *in situ* hybridization, Saddoris et al. (2009) showed that repeated exposure to a novel taste (sucrose) within the time frame required for temporal differentiation of Arc/Homer1a resulted in increased IEG activity, as well as increased overlap of activated neuronal populations in the GC. In addition, they showed that odor cues associated with sucrose, but not with water, elicited potentiation of IEG activity in the GC similar to that of sucrose itself, independently of Bla. Such cell populations, responsive to both CS (taste) and US (odor), are held to be critical for further plasticity.

Another study employed compartmental analysis of temporal gene transcription fluorescence *in situ* hybridization (catFISH) for Arc, relying on complete translocation of Arc from the nucleus to the cytoplasm over 30 min. This study, which applied Arc catFISH following a sucrose-conditioned odor preference test involving nine odor-taste pairings, showed, in contrast to the findings of Saddoris et al. (2009), that such a flavor experience paradigm induced a fourfold increase in the percentage of cells activated by both taste and odor stimulations in the Bla, but not in the IC (Desgranges et al., 2010). Furthermore, the authors showed that in odor-conditioned rats the number of cells responsive to one stimulus was unchanged. An earlier study, which used catFISH for Arc as well, also found that following CTA, specific Bla neuronal populations were responsive to both CS and US (Barot et al., 2008). Furthermore, this study demonstrated that when the LI-CTA paradigm was used, no coincident activation was detected. The identity of the cells expressing coincident activation remains to be further characterized.

BRAIN-DERIVED NEUROTROPHIC FACTOR

Brain-derived neurotrophic factor has emerged as a potent mediator both of synaptic plasticity on the cellular level, and of the interaction of an organism with its environment on the behavioral level (Moguel-Gonzalez et al., 2008). Along with its tyrosine kinase receptor TrkB, BDNF plays a critical role in activity-dependent plasticity processes, such as LTP, learning, and memory (Ma et al., 2011). Several studies have examined the effect of BDNF in the CTA paradigm. As mentioned above, BDNF-induced enhancement of CTA retention (Castillo et al., 2006), and this effect recently has been demonstrated to be dependent on activation of MAPK and phosphatidylinositol-3-kinase (PI-3K) in the IC (Castillo and Escobar, 2011). Furthermore, local administration of BDNF into the IC immediately after similar anisomycin administration performed prior to CTA training has been shown to reverse the anisomycin-induced CTA memory deficits almost to control levels. Taken together, these results imply that BDNF is a protein-synthesis-dependent memory enhancer (Moguel-Gonzalez et al., 2008).

Although BDNF is an IEG, the time scale of its mRNA expression is somewhat longer than those of Arc or Homer. For instance, BDNF mRNA expression in the rat IC peaked 6 h after CTA, and began to return to base level after 8 h (Ma et al., 2011). Surprisingly, this study found that BDNF levels in the Bla remained

unchanged, but an increase was observed in the central nuclei of the amygdala (CeA), peaking at 4 h. The authors showed that phosphorylated TrkB levels were elevated in the CeA long before the CTA-induced BDNF synthesis started, indicating a rapid activity-dependent BDNF release, presumably independent of protein synthesis. These data suggest that BDNF secretion and synthesis may be spatially and temporally involved in CTA memory formation. These authors also demonstrated that BDNF secretion and synthesis were necessary for STM and LTM, respectively, and, in addition, that BDNF injected into the CeA enhanced the CTA memory. Furthermore, in another study it was shown that a human naturally occurring polymorphic variant of BDNF in knock-in mice (Val66Met) caused impairments in CTA extinction, but not in its acquisition or retention, whereas in humans, homozygosity to this variant is associated with altered hippocampal volume, hippocampal-dependent memory impairment, and susceptibility to neuropsychiatric disorders. On the cellular level, this polymorphism was associated with alterations in intracellular trafficking and activity-dependent secretion of Wt BDNF in neurosecretory cells and cortical neurons (Yu et al., 2009).

FUTURE DIRECTIONS

In all, a more thorough understanding of the molecular mechanisms underlying taste memory and of the functioning of the corresponding regulatory processes in relevant neurons that subserve taste-learning processing circuits should help to elucidate many important basic aspects of neuronal function. The main future questions and the directions of future research might be more general in their nature, reflecting the potential development of knowledge and understanding in the field of biological mechanisms of learning and memory, or they might address questions specific to the taste systems in the mammalian/human brain.

For example, dissection of the temporal dynamics of any memory – acquisition/consolidation and the establishment of remote memory retrieval, relearning, and reconsolidation – which was outlined in **Figure 2** can be misleading. It is clear that acquisition and retrieval are well defined in time. However, the consolidation phase is defined mainly in negative rather than positive terms; it is sensitive to various disruptions at different time points following acquisition, and the connections between the various molecular entities involved in the process of taste learning are not well described. In **Figure 3** we outline some of the processes known to be taking place in the GC following novel taste learning. This off-line processing of information in the brain may represent a more continuous rather than a phasic process. However, the mechanisms and molecular/cellular participants in these processes are yet to be identified (**Figure 3**). It is clear that in order to better understand the consolidation or off-line processes, it is necessary to enhance the measurements of the biochemical factors that are correlated with and necessary for taste learning, and to improve their resolution to a few cubic micrometers or to cellular/subcellular levels. The tools for this kind of *in vivo* single-cell analysis have been dramatically improved recently, and will be used to obtain better descriptions of the molecular and cellular mechanisms underlying learning and memorizing processes. Moreover, we and others will aim to identify the specific circuit or neuronal ensemble involved in creation and maintenance of any given memory and its value.

Other aims, more specific to the taste system, will be to understand the neuronal mechanisms underlying the taste–memory condition of waiting "on hold" for many hours, with its physical and chemical information as a conditioned stimulus, pending arrival of the unconditioned stimulus or the digestive information that will enable the taste to be tagged as safe or dangerous. This specific and unique ability of taste-learning beautifully represents the flexibility of the neuronal system underlying learning and memory processes, and can also teach us about the limits of the neuronal systems abilities to create simple and associative memories.

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