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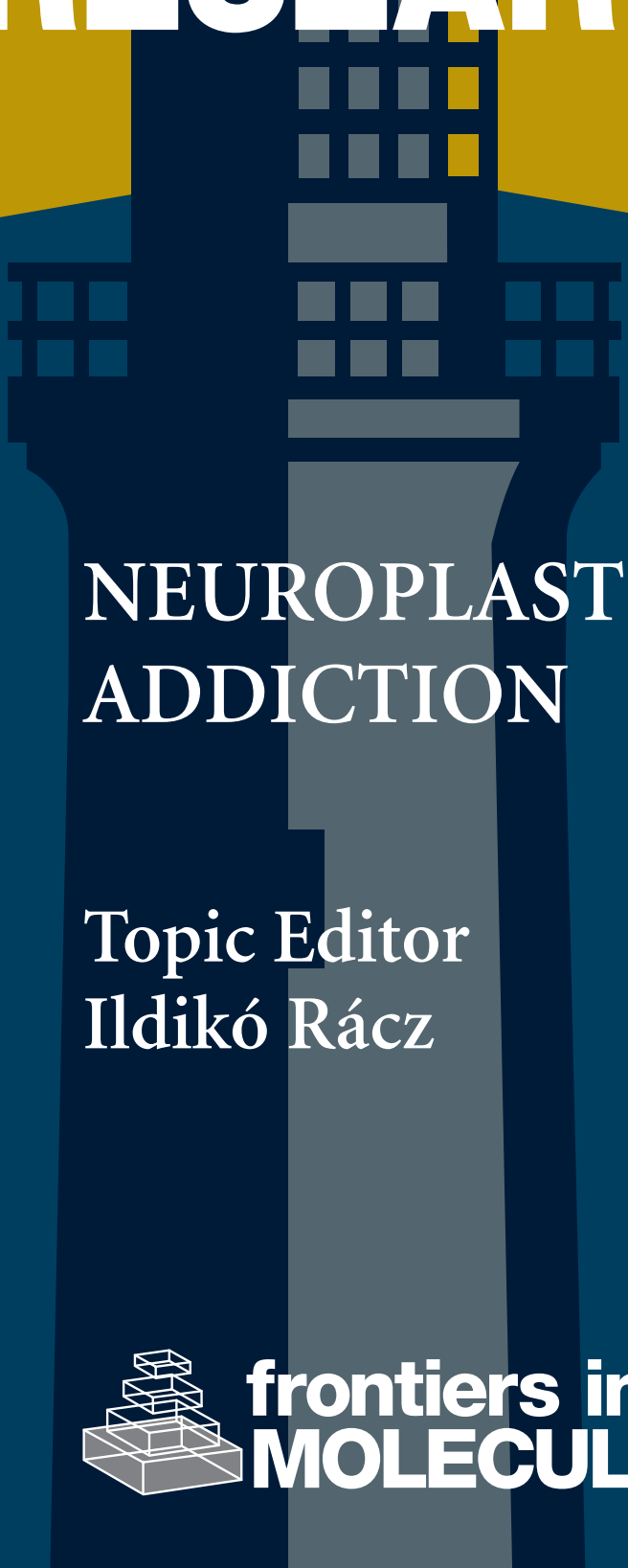

NEUROPLASTIC CHANGES IN ADDICTION

Topic Editor
Ildikó Rácz

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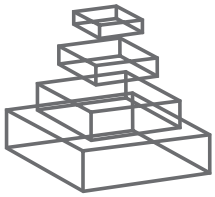

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NEUROPLASTIC CHANGES IN ADDICTION

Topic Editor:
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Addiction is a chronic relapsing disorder, which comprises impulsive and compulsive elements. Chronic drug consumption leads to long-term neuroadaptive changes in the brain thus result in an addictive state. However, development of addiction is a complex interaction between genetic, epigenetic and environmental factors. The resulting cellular and molecular changes mediate the transition from controlled drug use to the loss of control over drug-taking and drug-seeking.

The human association studies helped us to identify some important genetic factors responsible for the susceptibility to addiction. However, social, environmental circumstances highly influence the development of addiction. Using animal models helps us to examine the underlying neuronal/molecular processes under standardised conditions.

The aim of this Research Topic is to summarize our knowledge about the neuroplastic changes, which contribute to the maintenance of drug taking. Data presented in this Research Topic should also provide evidences how acute and long-term neuronal changes during withdrawal result in relapse. How different neuromodulators like endocannabinoids and endogenous opioids contribute to molecular mechanisms that mediate the transition from the controlled, occasional drug consumption to the uncontrolled, escalating drug use and seeking.

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Neuroplastic changes in addiction

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Drug addiction is a chronic, relapsing disorder, which is caused by many factors including genetic, epigenetic, environmental, and drug-related (Robison and Nestler, 2011). Loss of control over drug intake and compulsion for drug taking are the most characteristic features of addiction (Koob and Volkow, 2010). Although many individuals are exposed to substances of abuse, only subsets enter the addicted state. However, if the addicted state develops, it persists for life, suggesting that the underlying molecular changes in the brain are long-lasting. The present research topic was selected to highlight several new insights about genetic and drug-related factors that contribute to drug-induced neuroplastic changes.

Because dopaminergic cells play a pivotal role in the rewarding action of drugs of abuse, they were the starting point for the study of drug-induced synaptic alterations. Rodriguez Parkitna and Engblom (2012) added new insights to our knowledge about the synaptic plasticity of dopaminergic cells in the ventral tegmental area focusing on NMDA and AMPA receptor functions. For this, knockout mice with selective deletion of the NR1 subunit of the NMDA receptors were used. They examined NMDA receptor plasticity and burst firing activity, and found that this plays an important role in reward learning. Furthermore, they show NMDA receptors on dopaminergic cells are involved in drug-induced associative learning and in recall of drug-associated experiences.

Withdrawal is an unbalanced state characterized by increased stress, anxiety, and depression. During chronic drug consumption and withdrawal, the brain stress response system becomes dysregulated. The expression of several neuropeptides, including corticotropin releasing factor (CRF), neuropeptide Y, and dynorphin are stress-related. Furthermore, these neuropeptides are involved in the modulation of negative emotional states associated with drug addiction (Boutrel and De Lecea, 2008; Allen et al., 2011; Bruijnzeel, 2012).

Yadid et al. in their focused review (Yadid et al., 2012) concentrated on the neuroadaptive processes occurring during withdrawal. Evidence shows that endogenous opioid peptides β-endorphin, enkephalin, and dynorphin play an important role in substance reinforcement. β-endorphin and the neurosteroid dehydroepiandrosterone (DHEA) both modulate mood and drug addiction, and these modulatory functions are linked with each other. Application of exogenous DHEA-S (phosphorylated DHEA) into the nucleus accumbens elevated the level of extracellular β-endorphin. Thus, modulation of DHEA level in the brain may regulate extracellular β-endorphin levels which consequently controls stress coping including mood fluctuations. Together, these processes end up regulating the craving for drugs of abuse.

Dempsey and Grisel (2012) in their research paper examined the role of β-endorphin in the development of locomotor sensitization to repeated chronic alcohol exposure. They found that mice lacking β-endorphin did not develop locomotor sensitization to alcohol. These findings support the notion that β-endorphin modulates the locomotor effect induced by alcohol consumption and contributes to the neuroadaptive changes associated with chronic use.

Haass-Koffler and Bartlett (2012) in their review discussed the role of CRF in alleviation and maintenance of synaptic plasticity in the ventral tegmental area and amygdala. CRF facilitates the molecular changes induced by drugs of abuse like enhancement of glutamate-mediated excitation and reduction of GABA-mediated inhibition. Stress induces plastic changes in the limbic system that are thought to trigger the development of chronic anxiety and loss of control over limited drug use. Regulating stress processes by modulating the function of the CRF system may offer a possible new therapeutic approach in the treatment of relapse.

Besides the stress response, the Dynorphin/κ-opioid receptor (DYN/KOR) system is also highly dysregulated during chronic, excessive alcohol consumption and both contribute to the negative emotional state experienced during withdrawal (Koob and Volkow, 2010). Excessive alcohol consumption leads to the adaptation of the DYN/KOR system at the pharmacological, transcriptional, and epigenetic levels. Various key brain regions are involved via activation of different signaling pathways, like CREB/ΔFosB/BDNF, which contribute to altered downstream events. These changes can lead to escalated alcohol use, anxiety like behaviors, and sensitization following abstinence, which are the most common consequences of alcohol dependence (Sirohi et al., 2012).

Feduccia et al. (2012) in their review added new insights to the function of nicotinic acetylcholine receptors (nAChR) in alcohol and nicotine addiction. The nAChRs are ligand-gated ion channels which are wildly distributed in the brain and play a crucial role in synaptic neurotransmission (Mao et al., 2011). Both alcohol and nicotine are able to activate neuronal nAChRs. Activation of nAChRs by nicotine and alcohol facilitates and maintains long-term potentiation, long-term depression, and also structural changes in the hippocampus, amygdala, and mesolimbic dopaminergic system. Chronic nicotine treatment leads to up-regulation of nAChRs, which serves as a compensatory response to excessive receptor stimulation and is a main contributor to the

development of nicotine dependence. Several studies showed that chronic alcohol treatment induces the same processes and also that activation of nAChR function can reduce voluntary alcohol consumption.

Recently, a growing body of evidence shows that epigenetic mechanisms play a pivotal role in long-lasting changes in gene expression by regulation of transcriptional potential (McClung and Nestler, 2003; Chao and Nestler, 2004). Madsen et al. (2012) summarized our knowledge about the epigenetic modulation of gene expression induced by drugs of abuse. Self-administration of drugs of abuse induces transcriptional changes in the cell that represent a key mechanism affecting reward-related learning and further drug-related behaviors. Thus, voluntary drug intake controlled by a fine equilibrium of opposing molecular regulators can facilitate or inhibit compulsive drug use. This research has opened up new therapeutic strategies by modulation of transcriptional regulatory functions.

In her focused review, Kovacs (2012) summarized the role of neuroinflammatory processes in the development of chronic drug-induced molecular changes in the brain. Activation of microglia cells plays a pivotal role in drug-induced morphological, molecular, and physiological changes. These alterations involve release of proinflammatory cytokines, remodeling of synaptic functions, excitotoxic neurochemical changes, and phagocytic activity. Targeting microglia can serve as a potential new treatment strategy in addiction treatment.

Nylander and Roman (2012) provided a summary about the consequences of early-life stress. How changes early in life influence the function of peptide networks, like endogenous opioid peptides, oxytocin, and vasopressin later in adulthood. The results summarized in this review indicate that there is a strong association between early-life rearing conditions, opioids, and ethanol consumption. The effects of ethanol and also the treatment efficacy of opioid antagonists later in life are both dependent on early-life experiences.

Taken together, this research topic delivered new visions to our knowledge about the neuroplastic changes in chronic escalated drug consumption and in the following withdrawal.

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Addictive drugs and plasticity of glutamatergic synapses on dopaminergic neurons: what have we learned from genetic mouse models?

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Drug-induced changes in the functional properties of neurons in the mesolimbic dopaminergic system are attractive candidates for the molecular underpinnings of addiction. A central question in this context has been how drugs of abuse affect synaptic plasticity on dopaminergic cells in the ventral tegmental area. We now know that the intake of addictive drugs is accompanied by a complex sequence of alterations in the properties of excitatory synapses on dopaminergic neurons, mainly driven by signaling and redistribution of NMDA- and AMPA-receptors. It has, however, been unclear how these molecular changes are related to the behavioral effects of addictive drugs. Recently, new genetic tools have permitted researchers to perform genetic intervention with plasticity-related molecules selectively in dopaminergic cells and to subsequently study the behaviors of genetically modified mice. These studies have started to reveal how plasticity and drug-induced behavior are connected as well as what role plasticity in dopaminergic cells may have in general reward learning. The findings thus far show that there is not a one-to-one relation between plastic events and specific behaviors and that the early responses to drugs of abuse are to a large extent independent of the types of synaptic plasticity so far targeted. In contrast, plasticity in dopaminergic cells indeed is an important regulator of the persistence of behaviors driven by drug associations, making synaptic plasticity in dopaminergic cells an important field of study for understanding the mechanisms behind relapse.

Keywords: addiction, dopamine, genetically modified mice, glutamate, neural plasticity

INTRODUCTION

The intake of drugs of abuse initiates progressive molecular changes in different parts of the brain. Whereas, some of these changes are associated with addiction, others relate to physiological or behavioral phenotypes that are not critically involved in the development of persistent drug-induced behaviors. However, distinguishing the changes associated with addiction from those merely correlated with drug intake has remained a challenge. Here, we focus on drug-induced synaptic strengthening of excitatory synapses on dopaminergic cells, a drug-induced change identified with electrophysiological techniques, and discuss how modern genetic intervention techniques have been used to elucidate the function of such plasticity. First, we will provide a brief summary of the genetic tools used, highlighting their pros and cons, and subsequently, we will discuss results from studies in which they have been used to assay the function of synaptic plasticity on dopaminergic cells, both in the context of cocaine addiction as well as in the setting of natural motivated behaviors.

THE GENETIC TOOLBOX

Methods employed in the generation of genetically modified mice may be categorized based on the approach to introduce the mutation. The first approach is the replacement of a specific sequence in the genome by another, known as homologous recombination. This method involves transfection of embryonic stem cells with a DNA construct harboring a fragment that will replace the endogenous sequence flanked by homologous targeting sequences. The cells with recombined target sequence are then injected into mouse embryos in the blastocyst stage and transferred into a foster mother. Some of the offspring will be able to transmit the mutation, thus "founding" the mutated strain. This is the strategy employed in the generation of knockout ("KO") animals. It also allows for the introduction of special sequences such as loxP sites to intronic regions flanking critical parts of a gene (a "floxed" gene) (Abremski et al., 1983; Thomas and Capecchi, 1987; Gu et al., 1994). Homologous recombination is also used to replace parts of a gene with another sequence (a so called "knockin") such as a recombinase. Until recently, it was only possible to produce embryonic stem cells from specific mouse strains, such as 129 or FVB. Thus, many "KO" or "flox" lines have mixed strain background if they were not back-crossed over several generations. This may occasionally be a confounding factor, considering the differences in behavioral phenotypes of the 129 vs. C57 strains and, in particular,

their different sensitivities to reinforcement and learning abilities (Crabbe et al., 2010).

A second approach involves random insertion of a new DNA fragment (i.e., a transgene) in the genome. This is achieved by injecting a DNA construct into the prozygote (pronuclear injection) upon which a fraction of the offspring born from injected embryos will carry the transgene randomly, but stably, as it has been incorporated into their genome. This method has numerous applications (Branda and Dymecki, 2004; Dymecki and Kim, 2007). It is frequently used to introduce the Cre recombinase, an enzyme derived from bacteriophage P1, which belongs to the family of topoisomerases and has the ability to cut and ligate DNA strands (Abremski et al., 1983). Cre recognizes specific sequences, the loxP sites, which are not normally present in the murine genome. When a mouse with a recombinase transgene under the control of a cell-type specific promoter is crossed with an animal that contains a gene containing loxP sequences (created by homologous recombination), a deletion in the target gene will occur only in Cre-expressing cells (see **Figures 1**, **2**). For mouse lines generated by pronuclear injection, the milieu surrounding the site of integration may affect both the level of transgene transcription as well as the cell-type specificity of expression. This may be circumvented in most cases by introducing large transgenes (over 100,000 DNA bases) based on bacterial artificial chromosomes (BACs) in which long flanking sequences buffer the positional effects (Yang et al., 1997).

For studies on the functional role of plasticity in dopaminergic cells, it is of pivotal importance to be able to delete genes selectively in dopaminergic cells. This is typically performed with mouse lines expressing the Cre recombinase under the control of the promoter of the dopamine transporter (DAT), also called *Slc6a3*. The strategies employed differ considerably. The knock-in approach in which the Cre sequence is replacing one DAT allele was used to generate one of the frequently used strains (Zhuang et al., 2005; Zweifel et al., 2008). This approach is advantageous in that it typically offers high fidelity in the pattern of recombinase expression (i.e., only in dopaminergic cells). However, removing one allele of a gene is sometimes sufficient to produce a change in behavior or physiology, as indeed was reported in the case of DAT (Jones et al., 1998). In a second line, this problem was avoided by introducing the Cre recombinase after the DAT encoding sequence, separated by an IRES sequence to allow translation of both the DAT and the Cre sequence (Backman et al., 2006). In another mouse line, Cre was expressed from a large transgene (Parlato et al., 2006), as well as also using the CreERT2 modification (Rieker et al., 2011), which prevents recombination until animals are treated with the synthetic steroid tamoxifen (Feil et al., 1996; Engblom et al., 2008) (**Figure 1**). Finally, Cre has also been targeted to regions with dopaminergic cells using stereotaxic injection of viral vectors (Zweifel et al., 2008), thus limiting the recombination to cells infected by the virus. This method allows targeting of very precise brain areas with the mutation (i.e., only VTA instead of all DAT-expressing cells in the body). However, this approach is often limited by problems with recombination efficiency and limited selectivity (i.e., all neuron types instead of only DAT-expressing).

The reported efficiency of recombination by the DAT-driven Cre recombinases was excellent, affecting essentially all dopaminergic cells (Engblom et al., 2008; Zweifel et al., 2008; Luo et al., 2010; Rieker et al., 2011). Nevertheless, it was also reported that the offspring of an animal carrying the transgene and one floxed allele of the target gene would frequently show complete deletion of the allele (Δ/flox; so-called "germline" recombination). This could be due to transient activity of the DAT promoter during very early development or gamete formation. The frequency of such events was reported to be highest in mice where the Cre replaces one of the DAT alleles. To prevent the germ-line deletion from confounding the behavioral analyses, additional genotyping is performed to remove affected mice or control groups including heterozygous animals (i.e., Δ/flox without the Cre transgene) are added to the experimental design. Moreover, scattered Cre-mediated recombination is sometimes observed in brain areas not expressing DAT, as even transient activity of the transgene may be sufficient to drive the mutation. This type of activity has accumulating effects with age, often being more pronounced in older animals.

In plasticity studies, dopamine cell-specific deletions have mostly targeted NMDA receptors. These studies are based on partial deletions of the gene encoding the essential NR1 receptor subunit (*Grin1*) using modified variants of the gene with loxP sequences introduced in introns after exons 10 and 18 (Niewoehner et al., 2007), 10 and 23 (Zweifel et al., 2008), or 11 and 21 (Tsien et al., 1996). Deletion of molecules that are central for neural signaling, such as NMDARs, may cause compensatory changes in the activity of targeted neurons due to the recombination occurring early in the development of the neurons. In the case of the Cre/loxP-mediated ablations of NR1 in dopaminergic cells, which has already occurred in the second week of embryonic development, the mutation caused an increase in spontaneous activity of dopaminergic cells measured in slice preparations (Engblom et al., 2008; Zweifel et al., 2008). This problem could be circumvented by the use of the inducible CreERT2 variant previously described. However, as with all approaches described in this section, this method is not without pitfalls. First, so-called "leakiness," a level of recombination occurring without tamoxifen treatment, is a frequent problem. Second, tamoxifen treatment requires multiple i.p. injections over several days and is stressful to the animal, possibly causing persistent changes in behavior (Vogt et al., 2008).

In conclusion, although genetic intervention is an elegant way to test a gene's association with a specific phenotype, no method is without caveats. The discrete differences between superficially similar approaches should not be discarded as a technical aspect irrelevant to the conclusions. Inference of associations between genes and phenotypes is not possible without very extensive and sometimes impractical control experiments. In fact, it is simpler to demonstrate that a gene is not essential to a specific phenotype. Unfortunately, this is often avoided or unreported.

SYNAPTIC PLASTICITY IN DOPAMINERGIC CELLS

Synaptic plasticity, such as, long-term potentiation and long-term depression has received much attention since they may constitute cellular substrates of learning and memory (Malenka and Bear,

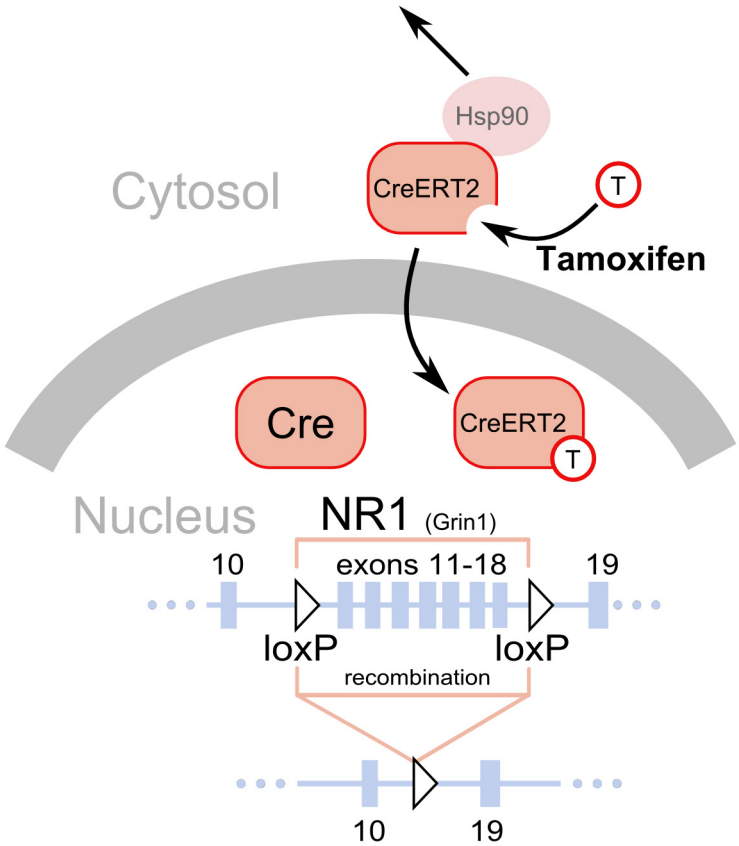

FIGURE 1 | Deletion of loxP flanked sequences by the Cre recombinase. A fragment of the NR1 (*Grin1*) gene is shown as a line with solid black rectangles representing exons. The numbers above the rectangles correspond to exons. The two triangles represent the loxP sequences introduced in introns, placed in the same orientation and flanking exons 11–18 (Niewoehner et al., 2007). The Cre recombinase has a nuclear localization signal and is normally shuttled to the nucleus after translation, where it catalyzes a deletion of the gene fragment flanked by the loxP sites. Thus, gene inactivation will occur soon after the gene promoter driving Cre expression becomes active, typically around the 13th day of embryonic development (Parlato et al., 2006). The CreERT2 is a fusion protein of the Cre and a modified ligand binding domain of the estrogen receptor (ERT2). The modification prevents binding of endogenous estrogens but allows binding of tamoxifen, a synthetic steroid (represented by a circle with a "T"). Additionally, the presence of the ERT2 domain enables interaction with the mechanisms normally responsible for keeping the estrogen receptor in the cytosol, in particular interaction with Hsp90. Binding of tamoxifen to the ERT2 releases its interaction with the cytosolic proteins and permits shuttling to the nucleus, where it catalyzes the deletion of the gene fragment flanked by loxP sequences.

2004). Because aberrant reward learning has been proposed to be a key feature of drug addiction, drug-induced synaptic plasticity is a strong candidate for being critical for the development and persistence of addiction and other related behavioral responses to drugs of abuse (Everitt et al., 2001; Hyman et al., 2006; Luscher and Malenka, 2011). Given the pivotal role of dopaminergic cells in the rewarding actions of addictive drugs, these cells were a natural starting point in the search for drug-induced synaptic alterations. In a landmark study, Ungless et al. (2001) showed that a single injection of cocaine leads to a strengthening of excitatory synapses on dopaminergic cells of the ventral tegmental area, measured as an increase in the AMPA/NMDAR ratio in slices from the midbrain of mice (**Figure 3**). Later on, it was shown that all major addictive drugs can induce the same adaptation and that it is also induced by stress and reward-predicting cues (Saal et al., 2003; Stuber et al., 2008). The synaptic strengthening lasts for approximately 5 days after a single passive injection of cocaine (Ungless et al., 2001) and at least 3 months after a period of cocaine self-administration (Chen et al., 2008). Given the relatively limited life span of the rat, this is a very persistent change and is therefore interesting from a clinical perspective. A schematic representation of main excitatory and inhibitory inputs to the VTA is shown in **Figure 4**.

On the molecular level, it seems that everything that leads to increased dopamine levels in the VTA triggers the synaptic strengthening. Such increases are due to the dendritic release of dopamine from the dopaminergic cells and can be triggered by an increased firing rate of the dopaminergic cells, which is sufficient for the synaptic plasticity to occur, as shown by an optogenetic approach (Brown et al., 2010), or through the local release of dopamine due to interference with catecholamine transporters in the VTA (Argilli et al., 2008). Thus, we know that cocaine administered to midbrain slices induces synaptic strengthening and that administration of a D1/D5 receptor antagonist, as well as

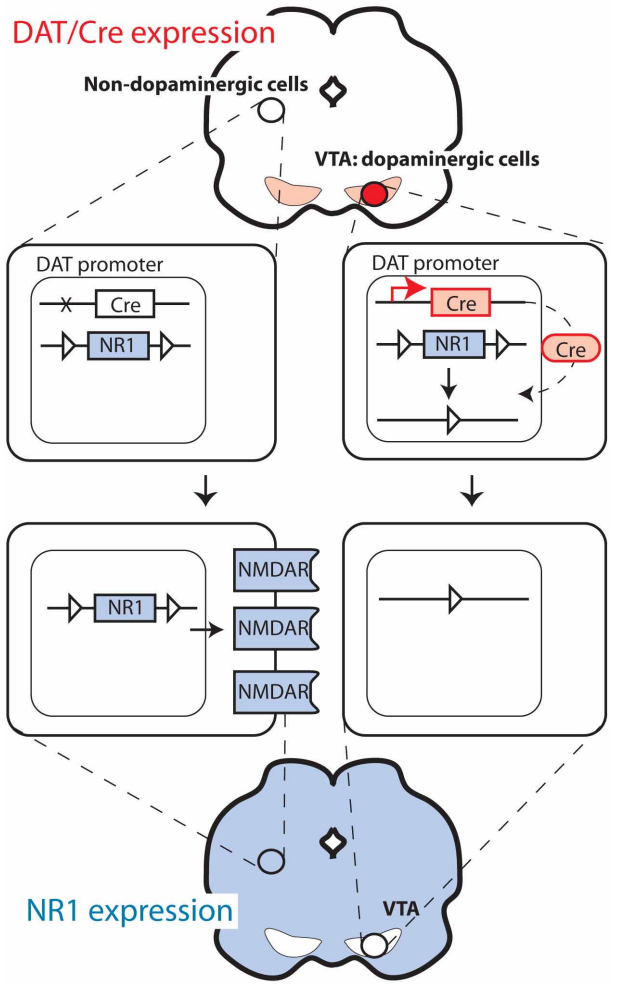

FIGURE 2 | Deletion of NMDA receptors selectively in dopaminergic cells.

the deletion of the D5 receptor, blocks the strengthening (Argilli et al., 2008). Because midbrain dopaminergic neurons express D5 but not D1 receptors (Khan et al., 2000), the dopamine action is most likely mediated by D5 receptors on dopamine neurons, although a role for D1 receptors on afferents contacting the dopaminergic cells cannot be ruled out. In any case, NMDA receptors are necessary for the plasticity. Because deletion of NMDA receptors selectively on dopaminergic cells in adult mice is sufficient to block the strengthening (Engblom et al., 2008; Zweifel et al., 2008), we know that this is due to the disruption of NMDAR signaling on the dopaminergic cells themselves and that it is unlikely that the loss of plasticity is explained by adaptations during development. Further, the AMPA receptor subunit GluR1, located on the dopaminergic neurons, is necessary for the plasticity (Dong et al., 2004; Engblom et al., 2008). Careful studies of the induction mechanism strongly indicate that drugs trigger the release of dopamine, indirectly leading to NMDAR activation on dopaminergic cells. This in turn leads to the redistribution of AMPA receptors so that receptors containing the subunit GluR2 are exchanged for receptors that do not (e.g., GluR1 homotetramers) (Bellone and Luscher, 2006) (**Figure 3**). These receptors have a higher conductance and are permeable to calcium, and their incorporation leads to an increased responsiveness and also changes the rules for further strengthening at the synapse (Mameli et al., 2011). The AMPA receptor redistribution is accompanied by a reduction in functional NMDA receptors at the synapse (Mameli et al., 2011). If drug use is discontinued, an mGluR1-dependent mechanism resets the synapse to the original setup after around 1 week (Mameli et al., 2007). In addition to changing the receptive properties of the dopaminergic cell, this plasticity triggers plastic events in the nucleus accumbens (Mameli et al., 2009). The role of drug-induced synaptic adaptations in this structure has been reviewed elsewhere (Thomas et al., 2008; Wolf, 2010; Luscher and Malenka, 2011).

To describe synaptic strengthening at these synapses as a unified phenomenon is, of course, somewhat simplistic. The dopaminergic cells in the VTA receive input from many different structures (**Figure 4**) and have different projections. Thus far, very little is known about how connectivity affects plasticity in these cells, but dopaminergic neurons projecting to the cortex have been shown to be molecularly distinct from neurons projecting to the nucleus accumbens and are known to react with synaptic strengthening in response to aversive rather than rewarding stimuli (Lammel et al., 2011). Complicating matters even further, the synaptic strengthening described above is not the only form of drug-induced synaptic plasticity in dopaminergic cells. Of particular interest for the interpretation of functional studies addressing the role of the synaptic strengthening is the form of plasticity called LTP-GABA (Nugent et al., 2007). This plasticity is NMDAR-dependent and increases the release of GABA from terminals contacting the dopaminergic cells. Interestingly, morphine, cocaine, and nicotine inhibit LTP-GABA (Nugent et al., 2007). A postsynaptic form of GABA plasticity, leading to weaker inhibitory transmission, has also been described after repeated cocaine exposure (Liu et al., 2005; Pu et al., 2006). Although, much less is known about these forms of inhibitory plasticity, it is important to keep them in mind when interpreting studies that intervene in the functioning of plasticity-related molecules in dopaminergic cells because, to some extent, they share mechanisms.

FUNCTIONAL STUDIES USING GENETIC TOOLS

As previously noted, a lot of drug-induced molecular changes have no impact on behavior, and because addiction is a behavioral disorder, it is of pivotal importance to understand how different forms of synaptic plasticity contribute to behavior. However, this task is not trivial, and the different approaches used all have their weaknesses. One source of information is classical pharmacological studies in which NMDA or AMPA receptor antagonists are injected into the VTA. These studies indicate that NMDARs and AMPA receptors in the VTA are essential for both cocaine-induced behavioral sensitization (Kalivas and Alesdatter, 1993) and conditioned place preference (Harris and Aston-Jones, 2003). However, the problem with these studies is that they also target non-dopaminergic cells in the VTA and

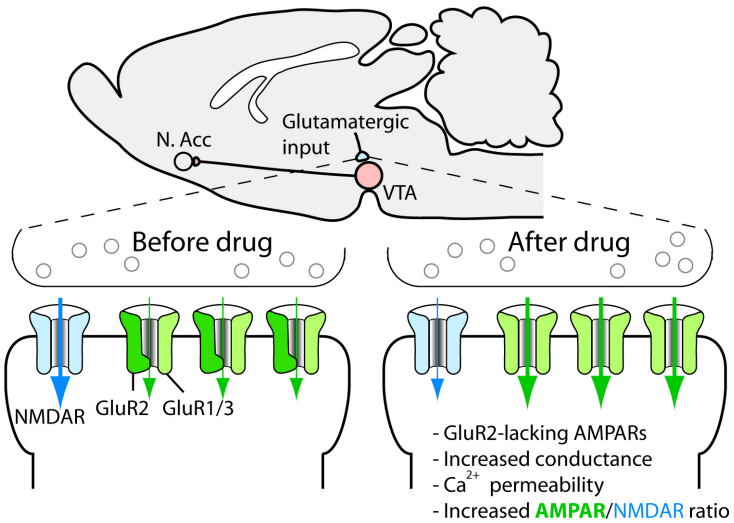

FIGURE 3 | Drug-induced synaptic strengthening of excitatory synapses on dopaminergic neurons.

that the effects observed are not necessarily due to inhibited plasticity as they could be due to the disruption of steady state signaling. Thus, the results could imply that NMDAR signaling in response to the drug is necessary for triggering some type of adaptation that is essential for sensitization, but this change could be something completely different from synaptic strengthening on dopaminergic neurons. In this context, an important technical addition was the use of rats with viral mediated overexpression of GluR1 in the VTA (Carlezon et al., 1997). These rats should have synapses that are inherently strengthened. Indeed, they showed an increase in the locomotor activity in response to an acute injection of morphine, indicating that they were in a pre-sensitized state. Additionally, these animals displayed a potentiated CPP to morphine (Carlezon et al., 1997). Recently, it was also shown that such rats show increased motivation for self-administration of cocaine (Choi et al., 2011). The main problem with this type of study is that the forced overexpression of GluR1 is not physiological. To an extent, this has been solved by loss-of-function approaches using mice lacking GluR1. In these mice, the basal properties of the excitatory transmission on dopaminergic cells seem to be relatively unaltered—most likely due to the function of GluR3 compensating for the lack of GluR1. However, the synaptic strengthening is blocked in GluR1 KO mice (Dong et al., 2004), showing that GluR3 cannot compensate for GluR1 in this aspect. Interestingly, the GluR1 KO mice formed a perfectly normal sensitization to cocaine, whereas conditioned place preference was affected in one study (Dong et al., 2004) but not in others (Mead et al., 2005; Engblom et al., 2008). The major limitation of this approach is that it is impossible to know if an effect on behavior is due to the lack of GluR1 in dopaminergic cells or elsewhere. This is not only a theoretical problem because *a priori*, it would seem quite likely that GluR1-mediated plasticity in the nucleus accumbens, the amygdala or the hippocampus would be involved in reward-learning-related behaviors. Recent studies using Cre/loxP methodology have added some missing pieces to this puzzle, although the results are not always easy to fit into a coherent picture. Importantly, some of these studies also looked beyond the early drug-induced behaviors using relapse models. In one study (Engblom et al., 2008), mice with deletions of GluR1 (GluR1-DATCre), GluR2 (GluR2-DATCre), or NMDARs (NR1-DATCre) specific to dopamine neurons were used. The fact that the deletions are selective to dopaminergic cells is important since the aim was to investigate the role of plasticity in these specific cells but also since mice lacking NMDARs in the entire body die within the first day after birth (Forrest et al., 1994). As expected, cocaine-induced strengthening of excitatory synapses on dopaminergic cells was blocked in mice lacking GluR1 or NMDARs on dopaminergic cells, whereas, this effect was intact in mice lacking GluR2. Intriguingly, mice lacking GluR1 or NMDARs showed perfectly normal locomotor sensitization and CPP, indicating that the synaptic strengthening is not important for these early drug effects (Engblom et al., 2008). In contrast, mice lacking GluR1 in dopaminergic cells showed a blocked extinction of the CPP, and mice lacking NMDARs in dopaminergic cells showed a normal extinction but a blocked drug-induced reinstatement of the CPP. In addition, when NMDARs were deleted in dopaminergic cells of adult mice, to avoid the synaptic scaling induced by early removal of NMDARs, an identical block of reinstatement was observed. The inducible NMDAR mouse line (NR1-DATCreERT2) was later also used in a self-administration paradigm, the results of which were compatible with a role of NMDARs in dopaminergic cells in relapse (Mameli et al., 2009). In this case, the mutant mice showed normal levels of cocaine self-administration and normal extinction behaviors but reduced cue-induced reinstatement of cocaine-seeking. Thus, although there are also conflicting results (Luo et al., 2010), both of these studies are compatible with the view that NMDAR-dependent synaptic plasticity on dopaminergic cells is important for the persistence of drug seeking rather than the early responses to cocaine. Intriguingly, in another study, Zweifel et al. found a blockade of cocaine CPP in mice with deletion of NMDARs in dopaminergic cells (NR1-DATCre) and

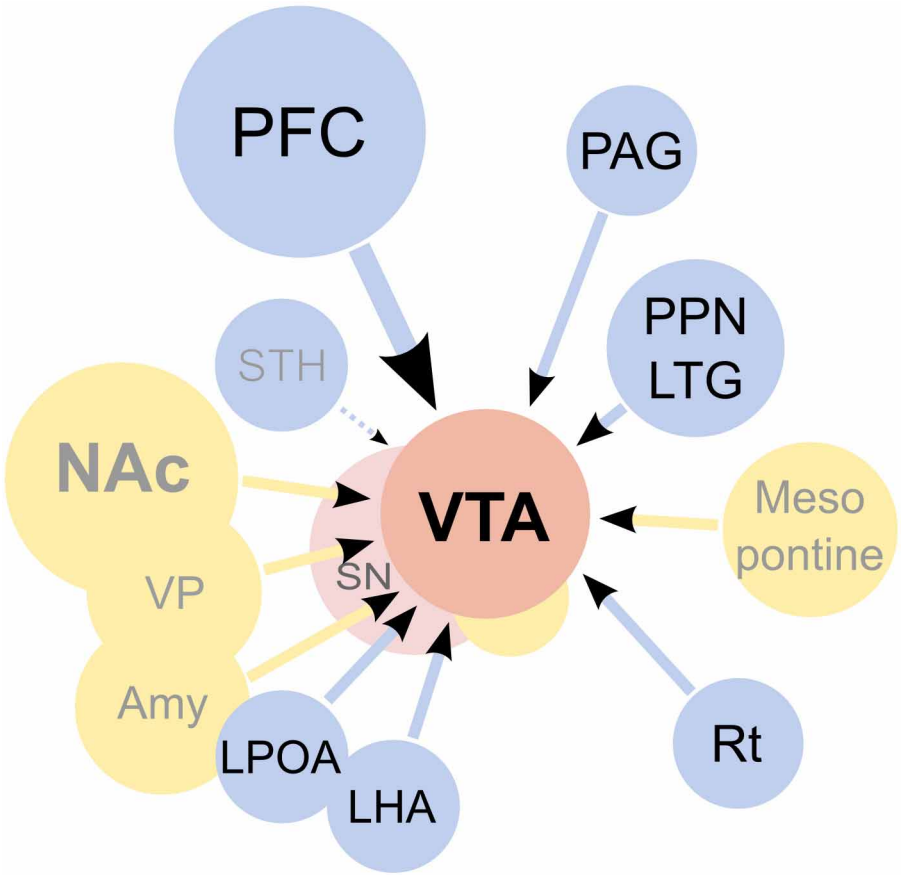

FIGURE 4 | Afferents to the ventral tegmental area (VTA). On the diagram, origins of the main excitatory inputs are shown as blue circles, origins of the main inhibitory inputs as yellow circles, and the VTA and substantia nigra (SN) as red circles. Excitatory inputs originate from the prefrontal cortex (PFC), periaqueductal grey (PAG), the pedunculopontine nucleus (PPN), the laterodorsal tegmentum (LTG), lateral hypothalamus (LHA), lateral preoptic area (LPOA) and the reticular formation (Rt). The subthalamic nucleus (STH) was reported to send projections to the SN but not the VTA. It should be noted that the excitatory projections are forming synapses not only on dopaminergic cells, but on other types of neurons present in the VTA as well. GABAergic signaling regulates the activity of the VTA, both through local inhibitory neurons as well as afferents coming from the nucleus accumbens septi (NAc), ventral pallidum (VP), globus pallidus (GP), amygdaloid nuclei (Amy) and the mesopontine rostromedial tegmental nucleus. Additionally, a fraction of the afferents from the PPN was found to be GABAergic. The diagram does not show all inputs to the VTA, notably omitting serotonergic and cholinergic afferents and also inputs to non-dopaminergic neurons. For an excellent review of the architecture of the mesolimbic system please see Sesack and Grace (2010).

in mice with a local deletion of NMDARs in the VTA using a viral approach (Zweifel et al., 2008). The latter could be due to an effect on non-dopaminergic cells because NMDARs on non-dopaminergic cells in the VTA have been shown to regulate other cocaine-induced responses (i.e., locomotor sensitization) (Luo et al., 2010), but the blocked CPP in the NR1-DATCre mice at first glance contrasts sharply with the unaffected CPP observed by Engblom et al. and another study using different mouse lines (Luo et al., 2010). The differing results might reflect the fact that Zweifel et al. used mice with knockin-based Cre expression, thus destroying one of the DAT loci, and with heterozygous global deletion of NR1, both of which are known to affect behavior. Another possible reason is that in the CPP procedure used by Zweifel et al., every conditioning is followed by a drug-free test, mimicking an extinction session and making the subsequent conditioning quite similar to a reinstatement session. Thus, the CPP protocol used by Zweifel et al. actually has similarities to the reinstatement protocol used by Engblom et al., possibly indicating a reason for the deficiency observed in these two tests. Thus, it is quite clear from these studies that NMDARs in dopaminergic cells are not a universal requirement for cocaine CPP, but the findings of Zweifel et al. together with data on natural reward learning (discussed later) and nicotine CPP (Wang et al., 2010) indicate that it may be important for some associations under specific conditions.

Collectively (summarized in **Figure 5**), the most solid conclusion from these studies seems to be that for cocaine, NMDAR-dependent signaling in dopaminergic cells is important in reinstatement of both CPP and self-administration, indicating a possible role in relapse. However, we know very little about the mechanisms behind this involvement. For example, we do not know at which stage they are required. Along this line, we also do not know if the blocked reinstatement has anything to do with the fact that drug-induced synaptic strengthening is blocked. It

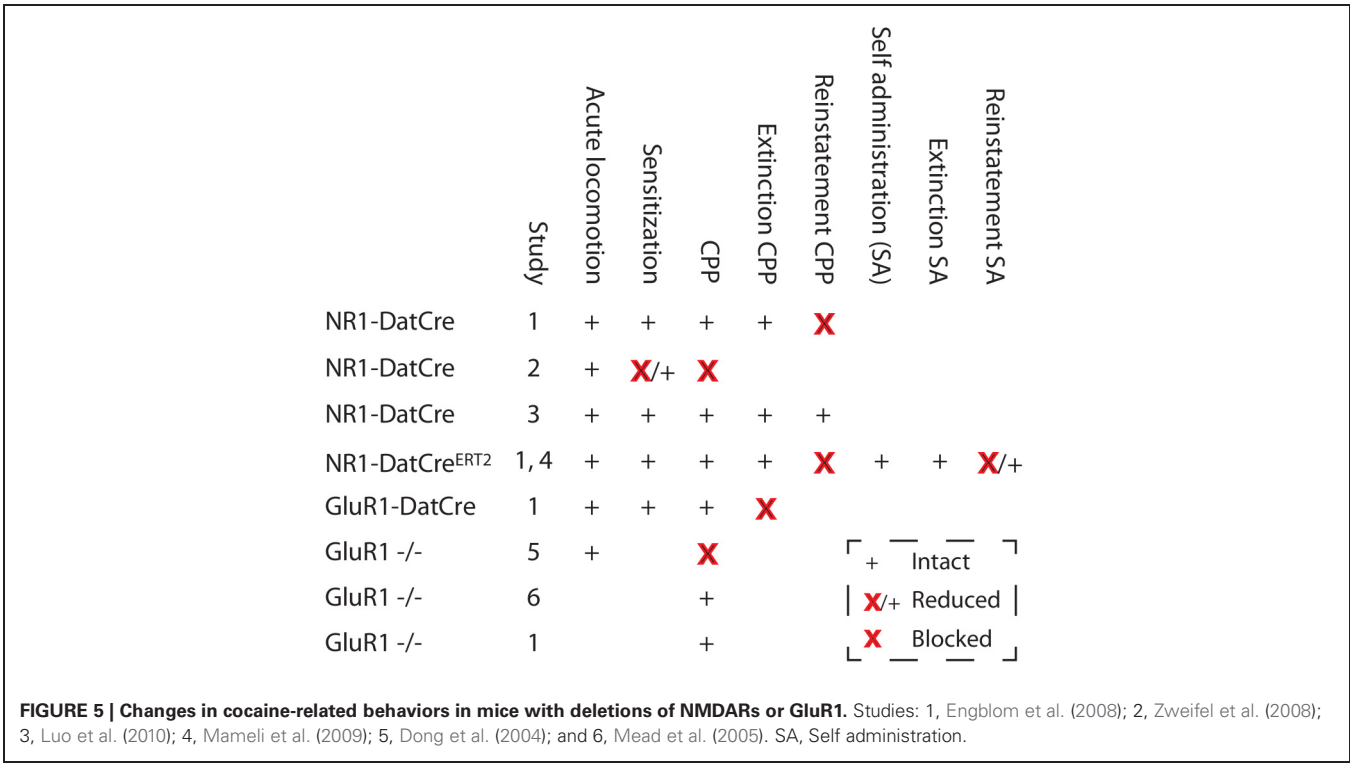

FIGURE 5 | Changes in cocaine-related behaviors in mice with deletions of NMDARs or GluR1. Studies: 1, Engblom et al. (2008); 2, Zweifel et al. (2008); 3, Luo et al. (2010); 4, Mameli et al. (2009); 5, Dong et al. (2004); and 6, Mead et al. (2005). SA, Self administration.

could be that impaired LTP-GABA explains the lack of reinstatement or even that NMDAR signaling is required only during the reinstatement procedure and has a role unrelated to any plasticity. Moreover, as will be discussed later on, the mice lacking NMDARs in dopaminergic cells have also been used as a model to study a lack of burst firing, another potentially important phenomenon in the context of reinstatement. Unfortunately, because the GluR1-DATCre mice showed a blocked extinction, it was not possible to determine if they reinstate, which would have provided strong support for the idea that deficiencies in synaptic strengthening in dopaminergic cells are responsible for relapse. Thus, we cannot, at the present stage, prove any one-to-one correlation between synaptic strengthening and a behavioral phenotype, which may not be surprising given how difficult it has been to determine the relation of other even more well-characterized types of plasticity. Nevertheless, the data clearly point to a role for synaptic strengthening in the persistence of cocaine seeking, even if the exact mechanism remains partly unclear.

THE ROLE OF NMDARs ON DOPAMINERGIC CELLS IN MOTIVATED BEHAVIORS

It was observed that inactivation of NMDARs diminished burst (phasic) firing of DA neurons, without notably altering tonic activity (Zweifel et al., 2009; Wang et al., 2011). The scaling of excitatory synapses on DA neurons in mutant mice had no significant effect on dopamine tissue or extracellular levels (Engblom et al., 2008; Zweifel et al., 2008, 2009, 2011; Mameli et al., 2009). Hence, the mutant mice are an excellent model to distinguish the roles of tonic and burst activity in learning and motivated behaviors. Indeed, loss of NMDARs on dopamine neurons did not influence several behaviors that are known to depend on tonic dopamine signaling. NMDAR loss did not alter novelty-induced locomotor activity and recognition of a novel object, food consumption when chow is available *ad libitum*, social behaviors or prepulse inhibition (Zweifel et al., 2009). Mutant mice had normal learning abilities, as indicated by a normal latency to find a hidden platform in the Morris water maze and an intact ability to navigate in a T-maze task (Zweifel et al., 2009). Strikingly, loss of NR1 on dopaminergic cells did not affect acquisition of the conditioned approach during Pavlovian training (Parker et al., 2010), a behavior that was shown to involve phasic activity of dopamine cells and NMDAR-dependent synaptic-plasticity in the VTA (Stuber et al., 2008). Despite the lack of phasic activity mutant mice responded to the presentation of a cue previously signaling delivery of a reward, and presentation of the cue elicited dopamine release similar to that observed in control mice.

Conversely, the lack of burst-firing of dopamine neurons in mutant mice impaired their performance in several tasks dependent on salient cues or contexts, which included the cued water maze and T-maze tasks as well as fear-potentiated acoustic startle (Zweifel et al., 2009). They were also slower to learn the instrumental task in food self-administration but showed similar motivation to obtain food as assessed by the progressive ratio test, where the number of instrumental responses necessary to obtain a food reward increases with each reward administered (Zweifel et al., 2009). Nevertheless, in other mouse strains with an equivalent ablation of NMDAR in dopamine cells no impairment in learning of an instrumental task rewarded with cocaine (Mameli et al., 2009) or food (Wang et al., 2011) was reported. To some extent these discrepancies could be explained by a deficit

of habit learning observed in mutant mice (Wang et al., 2011), which could affect their performance in tests with large numbers of repeated trials. Furthermore, differences in sensitivity to food vs. drug rewards were also observed in other behavioral tests. Mutant mice were found to have attenuated food CPP (Zweifel et al., 2009), even though most studies show that they develop a normal cocaine CPP (Engblom et al., 2008; Luo et al., 2010). Finally, the loss of NMDARs caused increased susceptibility to prolonged stress effects, as indicated by a persistently increased acoustic startle response and more anxiety-like behavior in the elevated plus maze test after aversive conditioning in mutant animals compared to controls (Zweifel et al., 2011). This phenotype could be prevented by injection of a viral vector that restored NR1 expression in dopamine cells, thus suggesting that impaired burst activity of VTA neurons may be involved in the development of anxiety disorders.

CONCLUSION

Reports on behavioral phenotypes of mice with selective NR1 ablation in dopamine cells show selective roles of NMDA receptor plasticity and burst firing activity in reward learning. Many behaviors previously presumed to be associated with NMDA receptor-dependent long-term potentiation in dopamine neurons, such as, drug-conditioned place preference, psychomotor sensitization, or drug self-administration, are normal or mildly altered in mutant mice. Conversely, the loss of functional NMDA receptors prevented reinstatement of cocaine-conditioned place preference, attenuated reinstatement of cocaine self-administration and also generally impaired behaviors dependent on salient cues or contexts. Thus, despite differences in reported phenotypes, a possible conclusion is that NMDA receptors on dopamine cells are involved in the recall of previously learned behaviors in response to salient stimuli.

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Modulation of mood states as a major factor in relapse to substance use

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Substance dependence is characterized by compulsive substance seeking and high vulnerability to relapse. A major challenge in current substance addiction research is not only to understand the immediate effects of substances of abuse on brain operations. It is also to define, at the behavioral and neural levels, how cognitive, emotional, and motivational processes interact with substance use in order to lead to this psychopathological state which defines addiction. For the last decade, research and progress into the biological basis of the addictive process has led to a rapidly growing number of pharmacological agents used to interrupt the progress of the addiction pattern, however without a significant/adequate impact.

It seems that the abstinent versus satiated states differ significantly (Kalivas and Volkow, 2005). Prolonged abstinence from substances of abuse is characterized by dysphoria, depression, and anxiety, coupled with high stress and craving; therefore strongly affecting the quality of life. It is speculated that memories of habitual substance use, produced by anxious and/or stressful emotional states, may have implications for understanding the role of learning and memory processes in substance addiction (Perrine et al., 2008; Packard, 2009). Substance dependent individuals, during their withdrawal, commonly employ thought-suppression to cope with stress and intrusive cognitions about the substance (Garland et al., 2012). Hence, abstinence-induced stress-related mood disorders are considered to be the main valence to define addiction as a chronic brain disorder, and stress is one of the major factors in substance seeking and relapse to its usage (Lu et al., 2003; Koob and Zorrilla, 2010).

Understanding the neurobiological basis of the abstinent state is a necessity to adequately treat substance relapse. The development of addiction and vulnerability to relapse following withdrawal is proposed to be the result of neuroadaptive processes within the central nervous system, leading to impairment in the mechanisms that mediate positive reinforcement and the emergence of affective changes (Weiss et al., 2001). A plethora of gene changes develop in the brain during chronic use or abstinence, which are related to the glutamate/corticoids, CREB/ERK, and NfκB pathways (Nestler, 2005; Li et al., 2008). Regardless the substance, a specific set of genes (Adora2a, Cnr1, Drd1, GPR88, Pde10a, Arpp21, Fam40b, Hpca, and Bc111b; mostly belonging to a huntingtin-centered pathway) were downregulated in the abstinent brain (Kalivas and Volkow, 2005; Le Merrer et al., 2012), hence possibly contribute to the negative affect characterizing protracted abstinence. Not surprisingly, these neuroadaptations, which occur during the addiction process, have been associated with multiple neuropsychiatric disorders (de Lecea et al., 2012).

Chronic stress increases the risk of depression, and is well known to increase relapse to drug seeking behavior (Bruchas et al., 2010). Depressive symptoms were suggested to be associated with abstinence-induced alterations in response to negative distracters (Froeliger et al., 2012). Findings suggest that the severity of depression symptoms are an important predictor of psychosocial treatment efficacy for cocaine dependence and, hence, underline the importance of adequately addressing depression symptoms to improve treatment outcomes (Stulz et al., 2011).

Serotonergic dysregulation in depression and addiction comorbidity was suggested as a novel target for the treatment of addiction and the prevention of drug relapse (Kirby et al., 2011). A few randomized clinical trials support the use of some antidepressant medications for combined cocaine dependence and depression (Rounsaville, 2004). Nonetheless, at the current stage of evidence, data do not unambiguously support the efficacy of antidepressants in the treatment of substance abuse/dependence (Pani et al., 2011). Notably, most negative results came from studies that evaluated selective serotonin reuptake inhibitors (SSRIs), while most positive results were found using norepinephrine/dopamine-reuptake-inhibitors, such as desipramine or bupropion. Although psychiatric symptoms are the prime motive of addicts requesting treatment, they are not always the expression of an associated mental disorder. Indeed, the presence of depressive/anxious symptomatology in the clinical presentation appears to be unnecessarily related to "dual diagnosis" (i.e., addiction and a mental illness). High-frequency abusers demonstrate an associated increased hypothalamic-pituitary-adrenal (HPA) axis activity, a characteristic stress response, to drug-cue exposure (Koob and Zorrilla, 2010). The role of the norepinephrine system in stress is well known, and its involvement in the mechanisms/potentiation of substance abuse has been explored (Belujon and Grace, 2011). Therefore, we suggest that noradrenergic antidepressants are effective in the treatment of substance relapse, since they initially control the stress circuit, and secondarily ease the depressive symptoms.

There is a preponderance of evidence that abuse of substances is parallel with stress disorders (Lu et al., 2003; Koob and

Zorrilla, 2010; Sinha, 2011; Moeller, 2012), and anxiogenic effects of abstinence from substances of abuse is dependent on the period of the withdrawal. The administration of anxiolytic agents, such as propranolol and buspirone or the 5-HT3 receptor antagonist ondansetron, after an abstinence period, reversed the anxiogenic effect induced by nicotine, alcohol, cocaine, and opiates (Costall and Naylor, 1992; de Oliveira Citó Mdo et al., 2012).

Several neuropeptides, including corticotrophin-releasing hormone, neuropeptide Y, hypocretin/orexin, nociceptin/orphanin have been shown to be stress-related. Not surprisingly, they also play a pivotal role in addiction, mediating the negative affect associated with stress (Boutrel and de Lecea, 2008; Bruijnzeel, 2012). Endogenous opioid-neuropeptides, such as β-endorphin, dynorphin, enkephalin, and others, have been shown to play a major role in substance reinforcement (Tang et al., 2005; Roth-Deri et al., 2008; Merenlender-Wagner et al., 2009; Wee and Koob, 2010).

Herein we wish to present a new piece to this puzzle, suggesting two putative candidates: the opioid neuropeptide β-endorphin and the neurosteroid dehydroepiandrosterone (DHEA), both shown to modulate mood and addiction.

β-endorphin is an endogenous opioid produced mainly in the arcuate nucleus of the hypothalamus, and released, in part, in the nucleus accumbens (NAc). β-endorphin induces euphoria and has rewarding and reinforcing properties (Roth-Deri et al., 2008). Consequently, it was demonstrated that cocaine induces dopamine-1-receptor dependent β-endorphin release in the NAc (Roth-Deri et al., 2008). Moreover, mice lacking β-endorphin demonstrated attenuation in cocaine-induced conditioned place preference (Marquez et al., 2008). β-endorphin binds with high affinity to μ- and δ-opioid receptors, while its affinity to the κ-opioid receptor is lower (Akil et al., 1984).

Using opioid receptor antagonists, several studies support a role for the μ-opioid type receptor in cocaine addiction in humans (Ghitza et al., 2010) and animal models (Kreek et al., 2009; Simmons and Self, 2009). Some studies showed that extended access to cocaine self-administration was associated with increased activity of the κ-opioid system or its endogenous agonist, dynorphin, in rats (Wee and Koob, 2010). κ-opioid receptor activation decreases acquisition or maintenance of cocaine, ethanol, morphine, and heroin by lowering their reinforcing/rewarding effects (Xi et al., 1998; Logrip et al., 2008; Wee et al., 2009). However, during lower sub-threshold doses of cocaine and morphine during maintenance, or abstinence following long access to cocaine, κ-opioid receptor activation facilitates substance self-administration or reinstatement, possibly through aversive-like and stress-like effects (Kuzmin et al., 1997; Wee and Koob, 2010). There is also some evidence for the involvement of the δ-opioid receptor in reward and addiction, but the studies are equivocal. Some have shown that non-peptidic δ-opioid receptor agonists elicit reward (Longoni et al., 1998), but some reported negligible abuse-related effects (Negus et al., 1998). The selective δ-opioid receptor antagonist naltrindole decreased responding for cocaine in rats, regardless of the schedule of reinforcement (Reid et al., 1995). Conversely, others (de Vries et al., 1995) reported that only a high dose of naltrindole, which decreased locomotor activity, attenuated cocaine self-administration. Intra-accumbal infusion of this δ-opioid receptor antagonist decreased cocaine self-administration, while administration into the VTA significantly increased cocaine-maintained responding (Ward and Roberts, 2007). Some demonstrated that withdrawal from cocaine, resulted in increased anxiety and depression, accompanied the desensitization of δ-opioid receptor function. Furthermore, cocaine-induced anxiety- and depressive-like behaviors were reversed by the δ-opioid receptor agonist SNC80 (Perrine et al., 2008).

Previous studies reported an attenuated β-endorphin response during ethanol or nicotine relapse. One study examined the extent to which β-endorphin response to stress is associated with early smoking relapse. The authors found that smokers who relapsed exhibited attenuated β-endorphin response to stressors, compared to those who maintained abstinence over the same period (Shaw and al'Absi, 2008). Moreover, smokers who underwent weekly exercise sessions had higher β-endorphin plasma levels and demonstrated a reduced smoking rate (Leelarungrayub et al., 2010). In another study, withdrawal from ethanol consumption led to decreased β-endorphin plasma levels. Chronic treatment with acamprosate, which increases β-endorphin plasma concentrations, caused a significant reduction in ethanol intake (Zalewska-Kaszubska et al., 2008).

Preliminary results elucidate a novel role for β-endorphin in incubation of cocaine craving, a model that evaluates abuse-related drug effects a long time after forced abstinence (**Figure 1A**). During the satiated state, β-endorphin levels correspond to substance self-administration. Associated cues throughout short-term withdrawal trigger elevated β-endorphin release (Roth-Deri et al., 2008). Throughout long-term abstinence, cues are unable to elicit enough β-endorphin release, concurrently with drug craving. Interestingly exogenous β-endorphin negated the heightened craving during long-term abstinence by acting on the δ-opioid-like receptor (unpublished results). Therefore, restoring abstinence-induced deficits in β-endorphin levels may be an important factor in preventing craving and maintaining abstinence.

We suggest that anxious-like and impulsive responses are linked to the compulsive seeking behavior observed after abstinence from substance abuse, through the δ-opioid receptor. By applying δ-opioid receptor agonists, we may bypass the lower efficacy of opioid-like receptor activity in the abstinence phase. Accordingly, this could cause a decrease in craving, simultaneously with decreased anxiety-like and depressive-like behaviors during extinction response.

Another candidate for intervention in substance abuse and relapse is DHEA, a natural steroid produced from cholesterol by the adrenal glands. DHEA is also produced in the gonads, adipose tissue, and the brain. It is structurally similar to, and is a precursor of, androstenedione, testosterone, and estrogens (Yadid et al., 2010).

Studies indicate that DHEA administration improves memory and cognitive processing; acts as a growth hormone in helping neurons grow new dendrites and controls levels of the stress hormone cortisol (Flood et al., 1988; Ulmann et al., 2009; Yadid et al., 2010). In healthy men and women, it was found that DHEA supplementation improved mood and sense of well being, including better quality of sleep, increased energy, relaxation, and higher capability of handling stress (Morales et al., 1994). Long-term treatment has been shown to modulate

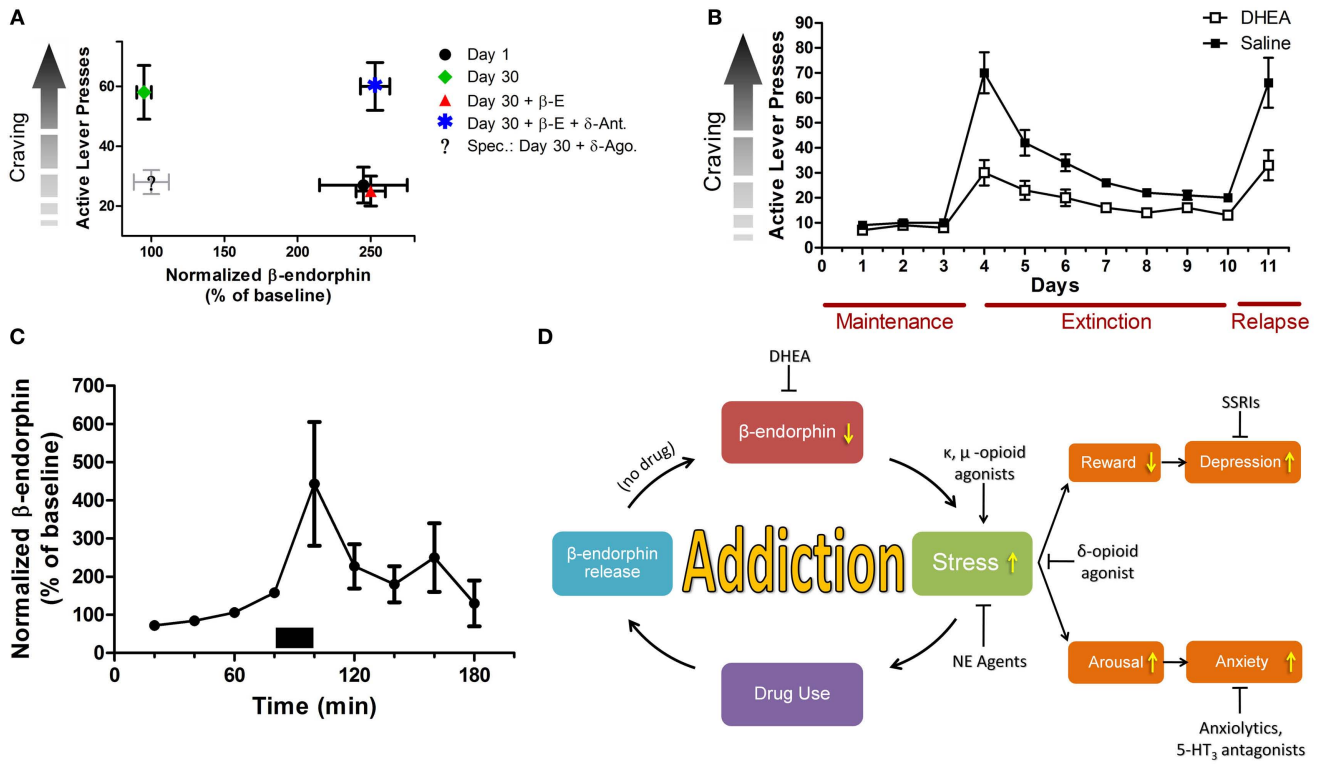

FIGURE 1 | Stress and mood in relation to substance addiction. (A) β-endorphin levels are inversely correlated with craving during prolonged abstinence. Rats were trained to self administer cocaine (0.75 mg/kg/infusion; 6 h/day; 10 days). Cocaine craving was measured by the number of active lever presses. β-endorphin levels were measured in the extracellular space of the n. accumbens, using the microdialysis technique. Craving was examined on day 1 or on day 30 of forced abstinence. Heightened craving is correlated with low β-endorphin release or low activity of the δ-opioid receptor in the n. accumbens. On withdrawal day 1 (black dot), β-endorphin levels are high and craving is low. On withdrawal day 30 (green rhomb), β-endorphin levels are low and craving is high. Adding β-endorphin (red triangle) markedly reduces craving, while a δ-opioid receptor antagonist (blue star) prevents the β-endorphin effect. Administrating a δ-opioid receptor agonist alone at 30-day abstinence is also expected to markedly reduce craving (legend: "Spec."). Note: β-E, β-endorphin; δ-Ant, δ-opioid antagonist; δ-Ago., δ-opioid agonist. **(B)** Effect of DHEA treatment or saline on extinction of cocaine self-administration. Rats were trained to self administer cocaine (1.5 mg/kg/infusion), after reaching stable maintenance, rats were either injected with DHEA or saline 90 min prior to placement in the operant chambers. After reaching abstinence, rats were reinstated with 10 mg/kg i.p. cocaine. DHEA significantly facilitates withdrawal and attenuates drug-induced relapse. **(C)** DHEA and β-endorphin are linked together. Using the reverse microdialysis technique, DHEA-sulfate (30 nM, marked by a bar) was applied into the n. accumbens, and β-endorphin levels in dialysates were measured. Perfusion of DHEA-sulfate causes a significant *in situ* transient increase in β-endorphin release. **(D)** A flow chart summarizing the role of stress and mood states in addiction. Note: stress is a core reinforcing component in addiction, and a major modulator of mood states. Hence, stress modulators that secondarily affect mood, strongly oppose relapse to substance usage. NE, norepinephrine; SSRIs, selective serotonin reuptake inhibitors.

distress, improve mood, and relieve depressive-like symptoms (Wolkowitz and Reus, 1999).

Recently it was successfully shown in an animal model of addiction that chronic exposure to exogenous DHEA markedly attenuated cocaine self-administration and decreased cocaine-seeking behavior of rats when applied during cocaine intake (Doron et al., 2006a) or during abstinence (**Figure 1B**). In another two preclinical studies it was found that DHEA attenuated reinstatement of cocaine-seeking behavior in rats (Doron et al., 2006b; Malkesman et al., 2006) and significantly increased neurogenesis (generation of newly formed neurons; Charalampopoulos et al., 2008; Yadid et al., 2010). In addition, the effect of DHEA was mimicked in heroin addicted rats (unpublished data) and humans (Maayan et al., 2008).

Interestingly, DHEA and β-endorphin are linked together. Application of exogenous DHEA-S (phosphorylated DHEA) into the NAc increases extracellular levels of β-endorphin (**Figure 1C**). Therefore, a lingering decease in brain DHEA supply may decrease extracellular β-endorphin levels, and this may signal lower capacity to cope with mood fluctuations and stress, that accompany the increase of substance craving.

These suggested candidates may represent a hallmark of drug abstinence and an adaptive mechanism in coping with a history of substance abuse disorders (**Figure 1D**). Prospective prolongation of DHEA and β-endorphin function may result in protracted drug withdrawal.

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Locomotor sensitization to EtOH: contribution of β-Endorphin

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Alcohol use disorders, like all drug addictions, involve a constellation of adaptive changes throughout the brain. Neural activity underlying changes in the rewarding properties of alcohol reflect changes in dopamine transmission in mesolimbic and nigrostriatal pathways and these effects are modulated by endogenous opioids such as β-Endorphin. In order to study the role of β-Endorphin in the development of locomotor sensitization to repeated EtOH exposure, we tested transgenic mice that vary in their capacity to synthesize this peptide as a result of constitutive modification of the *Pomc* gene. Our results indicate that mice deficient in β-Endorphin show attenuated locomotor activation following an acute injection of EtOH (2.0 g/kg) and, in contrast to wildtype mice, fail to demonstrate locomotor sensitization after 12 days of repeated EtOH injections. These data support the idea that β-Endorphin modulates the locomotor effects of EtOH and contributes to the neuroadaptive changes associated with chronic use.

Keywords: alcohol, EtOH, knockout, mu-receptor, opioid, transgenic

INTRODUCTION

Alcohol use disorders are a worldwide concern, devastating the health of individuals, families, and communities. Like virtually all disorders involving behavior, alcoholism results from a rich interaction of environmental influences expressed through genetic propensities, which makes it difficult to understand particular causal mechanisms. One strategy frequently employed by those seeking to elucidate biological substrates of complex traits is to utilize animal models in which simpler components of the phenotype are isolated and explored in a controlled laboratory setting (Crabbe, 2012).

One consequence of exposure to all addictive drugs is the ability to activate neural substrates involved in reward. At the heart of these circuits is the mesolimbic pathway which, when stimulated, leads to dopamine release in the nucleus accumbens. This pathway conveys information about stimulus salience. Another major dopaminergic circuit mediates locomotion, and all addictive drugs also stimulate movement. Thus, dopamine activity alerts an organism to important stimuli and motivates behavior; these pathways are both turned on by addictive substances (Robinson and Berridge, 1993; Kalivas and Volkow, 2005; Sanchis-Segura and Spanagel, 2006).

While the depressant effects of alcohol (EtOH) are widely appreciated, administration of lower doses, or analysis during the absorptive phase of blood-EtOH curve produce reliable stimulant effects, particularly in individuals susceptible to abuse and addiction (e.g., Wise, 1987; Phillips and Shen, 1996). Furthermore, chronic exposure to EtOH can result in sensitization to these changes, defined as an increase in behavioral stimulation following repeated administration, and this is also heritable. For instance, some inbred strains of mice are more prone to locomotor activation and sensitization than others (Phillips et al., 1995). Other strains of mice have been selectively bred to model these effects (Phillips et al., 1991; Crabbe et al., 1992). Moreover, studies have found genetic correlations between effects of EtOH on locomotor activity and measures of EtOH reinforcement (Malila, 1978; Phillips et al., 1998; Ponomarev and Crabbe, 2002).

At least one way that EtOH activates the neural pathways involved in reward and movement is through its ability to stimulate the synthesis and release of the opioid peptide β-Endorphin (β-E; Schulz et al., 1980; Gianoulakis, 1990, 2009; Scanlon et al., 1992; Przewlocka et al., 1994; Froehlich et al., 2000). β-Endorphin modulates dopamine activity in the mesolimbic pathway (Widdowson and Holman, 1992; Oswald and Wand, 2004; Zapata and Shippenberg, 2006; Jarjour et al., 2009) as well as in the nigrostriatal pathway (Willis, 1987; Boyadjieva and Sarkar, 1994; Sanchis-Segura and Aragon, 2002; Jarjour et al., 2009; Lam et al., 2010). Genetic differences in these opioid circuits correlate with a liability for alcohol use disorders in humans (i.e., Topel, 1988; Gianoulakis et al., 1989, 1996; Froehlich et al., 2000). In a series of studies, Sanchis-Segura and colleagues have made a strong case that β-E in the arcuate nucleus of the hypothalamus mediates EtOH induced locomotor activation and we and others have shown that low opioid activity compromises the reinforcing effects of EtOH (Grisel et al., 1999; Roberts et al., 2000; Racz et al., 2008).

Though the particular alleles and gene products contributing to EtOH induced locomotor changes remain obscure (Phillips et al., 1995), opioid peptides have been implicated (Prunell et al., 1987; Kuribara et al., 1991; Sanchis-Segura and Aragon, 2002; Sanchis-Segura et al., 2005). In this study we evaluated the effect of β-E on the development of locomotor sensitization to repeated EtOH exposure using transgenic mice that vary in their capacity to synthesize the peptide. "Knockout" (KO) mice have a premature stop codon inserted into their *Pomc* gene and therefore fail to produce β-E. These mice are fully backcrossed onto the

C57BL/6J strain, which provide a useful comparison along with heterozygote (HET) mice that have 50% of control levels of the endogenous opioid.

MATERIALS AND METHODS

SUBJECTS

Subjects were adult naïve male and female wildtype controls (C57BL/6J; B6), β-E deficient (B6.129S2-*Pomc*^{tm1Low}/J; KO), and heterozygous (HT) mice. Transgenic mice were developed over a decade ago in the laboratory of Malcolm Low (Rubinstein et al., 1996) by insertion of a premature stop codon into the *Pomc* gene and have been fully backcrossed onto the B6 genome. Homozygotes (KO) cannot synthesize β-E, though all other *Pomc* products show normal expression. Opioid receptor expression also remains unchanged (Rubinstein et al., 1996). HT mice produce 50% of B6 levels of β-E.

Mice for these studies were bred from isogenic pairs derived in-house from HT progenitors purchased from Jackson Laboratories (Bar Harbor, ME, USA). The gene mutation has been fully backcrossed to the C57BL/6J strain (>20 generations). Mice were group housed by sex with 4–5 per Plexiglas cage following weaning at 20–21 days and maintained in a at 21 ± 2°C colony room on a reverse 12:12 light:dark cycle with lights on at 7 PM. Water and food were available *ad libitum*.

EXPERIMENTAL PROTOCOL

We followed the method developed in Tamara Phillips' lab (Lessov et al., 2001) in which C57BL/6J mice evince robust locomotor sensitization following repeated administration of EtOH, although these investigators suggests that at least some of the increased locomotor activity on test day may reflect a "novelty response" (Meyer et al., 2005) since in this paradigm the mice have not been in the test chamber for several days before the sensitization measure is taken.

On Days 1–3 and 14 of the two-week protocol, mice were assayed during the dark phase of their light/dark cycle in a Plexiglas open field arena (50 cm^3) equipped with infrared sensors and coupled to Tru Scan software (Coulbourn Instuments, Whitehall, PA). Horizontal distance traveled and the number of rears (two front feet off the ground) was assessed for each mouse during the 10 min test period on Day 1–3 and 14. The cage floor was thoroughly cleaned between experimental subjects with non-toxic, low-odor solution, and testing order was randomized across genotypes.

On Days 1–3 animals received injections and were placed in the testing arena for 10 min. On Day 1 and Day 2 all animals received saline but on Day 3, two groups of animals—designated chronic or acute EtOH (CE or AE) received 2.0 g/kg EtOH instead of saline. Days 4-13, animals were injected and then immediately placed back in their home cages; there was no measurement of locomotor activity. The CE group got 2.5 g/kg EtOH each of these days, and the AE and chronic saline (CS) groups got equivolume saline. On Day 14, all animals received 2.0 g/kg EtOH and locomotor activity was evaluated for 10 min. All injections were delivered intraperitoneally (i.p.) and EtOH was administered in a 20% (vol:vol) solution in saline. All procedures were carried out in accordance with the National Institutes of Health guidelines and approved by the Animal Care and Use Committee of Furman University.

STATISTICAL ANALYSIS

Data were analyzed using a three factor ANOVA with genotype (three levels: B6, HT, and KO), experimental condition (AE, CE, and CS), and sex for main effects, on days 1–3 and 14 separately. In addition, a repeated measure ANOVA was conducted across days by strain and condition (excluding sex). Significant interactions were further examined using Fisher's least significant difference (LSD) test. Statistical analyses were performed using SPSS Statistics 17.0. The criterion for significance was set at $p \leq 0.05$.

RESULTS

There were main effects of sex on virtually every measure, as females are well known to have more locomotor activity under basal conditions as well as following EtOH administration (e.g., Lynn and Brown, 2009; Tayyabkhan et al., 2002). However, in this overall analysis, there were no significant interactions involving sex and genotype—i.e., the strain differences were not dependent upon sex, and therefore data were collapsed across sex for analysis.

In the repeated measures ANOVA there was a main effect of strain on horizontal distance traveled [$F_{(2,\ 91)} = 7.265$, $p < 0.001$] but no main effect of experimental condition [$F_{(2,\ 91)} = 2.25$, $p > 0.05$] or interaction between these two variables [$F_{(4,\ 91)} = 1.002$, $p > 0.05$]. There was also a main effect of test day $F_{(3,\ 273)} = 94.306$, $p < 0.001$, interactions between strain and day $F_{(6,\ 273)} = 10.584$, $p < 0.001$, and strain and experimental condition $F_{(6,\ 273)} = 3.634$, $p < 0.01$ as well as a triple interaction between strain, day and drug condition $F_{(12,\ 273)} = 2.061$, $p < 0.05$. While B6 mice in the CE condition (both sexes) showed sensitization, neither of the β-endorphin deficient groups did (**Figure 1**).

There was no difference in distance traveled or rears on Day 1 across either lines or drug groups (though all received saline on Day 1) and no interactions. With one exception the results were the same on Day 2 as on Day 1: there was no difference in distance traveled across genotypes or experimental groups and no interactions. However, there was a significant effect of genotype on rears on Day 2 in that KO did not habituate as readily as either B6 or HT mice [$F_{(2,\ 91)} = 7.911$, $p < 0.01$; data not shown].

On Day 3, there was a significant effect of genotype on distance traveled [$F_{(2,\ 91)} = 6.377$, $p < 0.01$], evincing a direct relationship between β-E levels and horizontal distance traveled, but in this Two-Way ANOVA with 3 treatment groups and 3 genetic lines, there was no main effect of treatment or interaction between treatment and genotype. However, because the AE and CE groups were treated identically and injected with 2.0 g/kg EtOH, they were collapsed into a general "EtOH" group and compared to saline-treated subjects in a separate 2-factor ANOVA (this time with only two levels of treatment) evaluating locomotion on Day 3 (horizontal distance in cm). As shown in **Figure 2**, B6 tended to be stimulated by EtOH, while KOs were sedated by EtOH and HTs were intermediate. This is substantiated in the statistical results in which there was a main effect of strain:

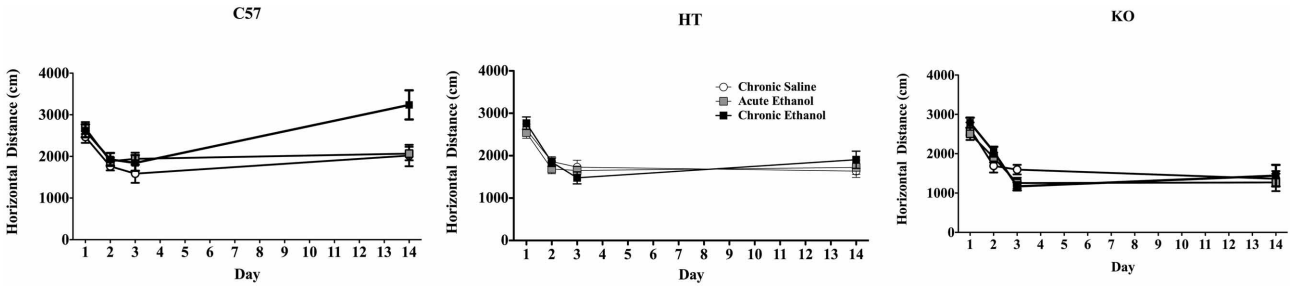

FIGURE 1 | Locomotor activity in mice during four, 10 min test periods on Days 1–3 and 14 of a 2-week experimental protocol. On Days 1 and 2 all mice received intraperitoneal saline injections, on Day 3 the chronic saline (CS) group received saline again, but all other subjects received 2.0 g/kg EtOH. On Days 4–13, half of these were administered 2.5 g/kg EtOH (Chronic EtOH—CE) and the remainder was given equivolume saline (Acute EtOH—AE, and CS) and all were placed in their home cages immediately following injection. On Day 14, all animals were administered 2.0 g/kg EtOH and evaluated in the activity chambers. The *left panel* shows the horizontal distance traveled in cm (data show mean ± SE) of adult male and female C57BL/6J mice, demonstrating the development of locomotor sensitization in CE-treated subjects. The *middle panel* shows analogous experimental groups of heterozygote (HT) mice and the *right panel* shows the same data in β-E deficient (KO) mice. Neither of these latter two groups developed locomotor sensitization.

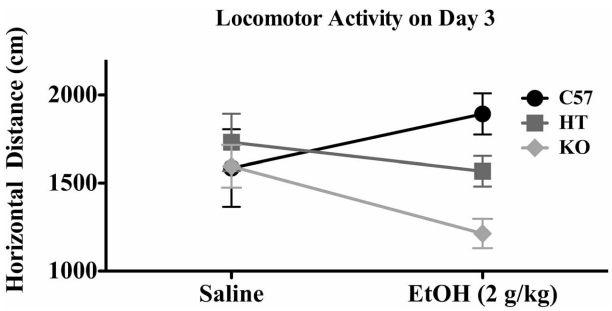

FIGURE 2 | Horizontal distance traveled in mice of each genotype on Day 3 of the experimental protocol. Mice were either given 2.0 g/kg EtOH by intraperitoneal injection or equivolume saline immediately before 10 min evaluation (see text for experimental detail). Data represent group means ± SE.

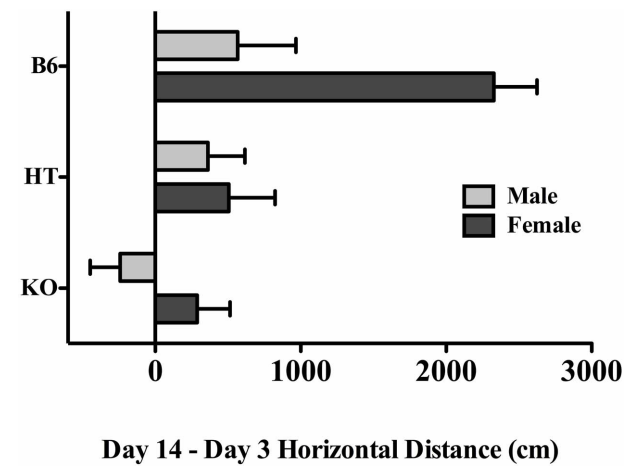

FIGURE 3 | The difference in horizontal locomotor activity between the first and last exposure to EtOH in chronically treated mice across the three genotypes and both sexes (see text for experimental detail). Data represent group means ± SE.

$[F_{(2,\ 91)} = 3.750,\ p < 0.05]$, but not of drug $[F_{(1,\ 91)} = 0.593,\ p > 0.05]$ but there was an interaction between strain and drug $[F_{(2,\ 91)} = 3.750,\ p < 0.05]$. While there was no effect of genotype on rears, there was an effect of EtOH $[F_{(2,\ 91)} = 166.636,\ p < 0.001]$ in which the drug generally decreased rearing activity, but this measure on Day 3 did not depend upon strain.

On Day 14 there were differences between genotype in the distance traveled $[F_{(2,\ 91)} = 20.356,\ p < 0.001]$, differences across treatment groups $[F_{(2,\ 91)} = 4.222,\ p < 0.05]$, and a significant interaction between genotype and treatment group $[F_{(4,\ 91)} = 2.961, p < 0.05]$: post-hoc analysis indicated sensitization only in B6 mice (in the CE group; see **Figure 1**). There were no differences in rears between strain or treatment groups and no significant interaction between these two measures on Day 14.

In order to directly compare the magnitude of locomotor sensitization that developed across the experimental period while taking into account the genotypic differences on Day 3 (see above) we conducted a 2-way (strain and sex) planned comparison within the CE treatment groups, using a difference score that was calculated by subtracting horizontal activity on Day 3, after the first exposure to EtOH, from Day 14 activity, following the chronic regimen. Here, the effect of genotype was significant with $F_{(2,\ 29)} = 9.260$, $p < 0.001$, indicative of a positive correlation between β-E levels and locomotor activity on Day 14. The post-hoc test revealed that B6 mice differed from either of the β-E deficient lines. There was, as in the overall analysis, a main effect of sex $[F_{(1,\ 29)} = 8.726,\ p < 0.01]$, but also, a (just) significant interaction between strain and sex $[F_{(2,\ 29)} = 3.312,\ p = 0.051]$. These change scores are depicted separately by strain and sex in **Figure 3**, where evidence of locomotor plasticity is more evident in wildtype females than in all other groups.

DISCUSSION

Mice deficient in β-Endorphin demonstrate a blunted locomotor response to acute alcohol, and also fail to develop locomotor sensitization after 12 days of daily drug administration. Though

there were no observable differences in activity under baseline conditions (following injection with saline) 2.0 g/kg EtOH moderately stimulated locomotor activity in C57BL/6J mice but depressed it in mice lacking β-E. Furthermore, while repeated injections of EtOH led to locomotor sensitization in C57BL/6J mice, transgenics with either low or absent β-E showed no evidence of this plasticity. These data replicate findings by others showing the development of sensitization in this inbred strain (e.g., Lessov et al., 2001; Tarragón et al., 2012) and extend them by suggesting that β-E plays a critical role in this change as transgenic subjects unable to synthesize β-E, but otherwise virtually identical to controls, fail to demonstrate this plasticity.

Drug sensitization is thought to underlie changes associated with alterations in the reward pathway such as drug-induced conditioned place preference, operant self-administration and other forms of drug seeking (Vezina, 2004). Indeed, a empirical evidence and theoretical explorations link the neural plasticity underlying locomotor sensitization to the behavioral characteristics of drug abuse including drug seeking, compulsive use, and relapse (Robinson and Berridge, 1993, 2008; Kalivas et al., 2005). Thus, many have argued that behavioral sensitization to the locomotor effects of drugs provides an index of neural changes mediating the dependent state (Robinson and Berridge, 1993; Kalivas and Volkow, 2005; Sanchis-Segura and Spanagel, 2006). The present findings support the contention that the opioid peptide β-E plays a critical role in the neural substrates of alcohol reinforcement and addiction. Along this line, EtOH self-administration in animals depends upon this peptide (Grisel et al., 1999; Williams et al., 2007; Racz et al., 2008; but also see Grahame et al., 1998) and clinical studies have associated β-E levels with liability toward alcohol use disorders (Gianoulakis et al., 1989; Wand et al., 2001; Zalewska-Kaszubska and Czarnecka, 2004).

As with all behavior, the neural mechanisms of sensitization are complex and multidimensional. Though the current study, along with previous reports (Camarini et al., 2000; Pastor and Aragon, 2006; Abrahao et al., 2008; Tarragón et al., 2012) strongly implicates endorphins, these peptides are surely not acting alone. For instance, both endorphins and endomorphins are highly efficacious agonists at μ receptors, and though these receptors appear to be unchanged in transgenic mice (Rubinstein et al., 1996), other opioids may also contribute. Moreover, evidence supports the involvement of various other neurotransmitters including amino acids (i.e., γ-aminobturic acid and glutamate) and monoamines, in this plasticity (Broadbent et al., 1995; Chester and Cunningham, 1999; Meyer and Phillips, 2007; Carrara-Nascimento et al., 2011). Repeated EtOH administration has also been linked to activation of the Hypothalamic Pituitary Adrenal (HPA) axis and shown to be dependent upon the neuroendocrine response to stress (Roberts et al., 1995; Pastor et al., 2008, 2012).

β-E is involved in a wide array of behaviors, including many of those associated with analgesia, reward, attachment, and stress. While activation of the stress (HPA) axis leads to synthesis and release of β-E, this peptide plays a role in endocrine and behavioral allostasis. Exposure to a stressor induces the hypothalamus to secrete corticotropin releasing hormone (CRH) in the adenohypophysis, mounting a neuroendocrine response, and leading to activation of the sympathetic nervous system and behavior. Upon stimulation by CRH, the anterior pituitary turns on *POMC* transcription to stimulate synthesis of adrenocorticotrophin hormone (ACTH) and β-E (among others). ACTH leads to the synthesis and release of glucocorticoids from the adrenal gland but may also inhibit *POMC* activity (Suda et al., 1988, 1993). Negative feedback is typical in the stress response, and virtually every stress-induced chemical change subsequently contributes to dampening further HPA activation. For example, corticosterone (in rodents) or cortisol (in humans), through interaction with a dense population of glucocorticoid receptors in the brain, suppresses HPA activity. Blockade or deletion of either CRH or glucocorticoids prevents the acquisition of EtOH-induced locomotor sensitization (Roberts et al., 1995; Pastor et al., 2008, 2012) suggesting that an intact neuroendocrine response to stress is necessary to exhibit locomotor sensitization to EtOH.

These data are somewhat contradictory because, although synthesized and released in response to stress, β-E inhibits the stress axis by counteracting CRH synthesis in the paraventricular nucleus of the hypothalamus (Buckingham, 1986; Plotsky, 1991; Hunt and Zakhari, 1995; Janssen and Arntz, 2001; Amat et al., 2005; Ribeiro et al., 2005). Thus, low or absent β-E is associated with exaggerated neuroendocrine and behavioral responses to stress (Buckingham, 1986; Grisel et al., 2008; Barfield et al., 2010) and disruptions in coping behavior (Hunt and Zakhari, 1995; Gianoulakis, 1998; Barry and Grisel, 2012). We have shown, i.e., an inverse relationship between β-E levels and anxious behavior, as well as adrenal weight, in these mice (Grisel et al., 2008). Since low β-E compromises the ability to manage stressful stimuli (Gianoulakis, 1998; Sarkar et al., 2007; Barfield et al., 2010) one might expect augmented, rather than attenuated, locomotor sensitization in β-E deficient mice. However, because CRH mediates the EtOH-induced increase in β-E (Gianoulakis, 1998; Lam and Gianoulakis, 2011) perhaps the low CRH activity indirectly affects sensitization, through a consequent blunting of the β-E surge following EtOH administration.

It is well documented that acute exposure to EtOH, like exposure to stressors, causes the synthesis and release of β-E. The relationship between opioids and EtOH-induced locomotor changes has been extensively studied by Carlos Aragon and his colleagues, in over two decades of research. Early studies implicated the opioid system in the effects of stress and EtOH on movement (Aragon et al., 1990; Trudeau et al., 1991). Fifteen minutes of restraint stress decreased locomotor activity, but this effect of stress was blocked (in an opioid-dependent manner) by EtOH pre-treatment. These data fit with the recent findings (Pastor et al., 2012) that CRH and corticosterone are necessary to evince EtOH-induced locomotor changes in mice. However, this group also showed that either pharmacologic antagonism of μ-opioid receptors or lesioning endorphinergic neurons in the hypothalamus prevents EtOH-induced increases in locomotor activity and other forms of adaptation including conditioned place preference (Sanchis-Segura et al., 2000;

Sanchis-Segura and Aragon, 2002; Pastor et al., 2004, 2005; Pastor and Aragon, 2008). The endogenous opioid system has been implicated in several aspects of the rewarding and addictive actions of ethanol. Modulation of the mesolimbic dopamine system by β-E contributes to positive reinforcement following drug administration (see Roth-Deri et al., 2008 for review). Individual variation in the β-E response to EtOH has been used to explain differences in the liability toward high consumption and abuse (Gianoulakis, 1996, 1998; Froehlich et al., 2000; Dai et al., 2002, 2005). The current study, demonstrating a failure to develop locomotor sensitization in an animal model of endorphin deficiency, adds to the growing body of pre-clinical research suggesting that β-E modulates the neuroplasticity underlying chronic changes in behavior associated with the development of alcohol addiction.

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Stress and addiction: contribution of the corticotropin releasing factor (CRF) system in neuroplasticity

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Corticotropin releasing factor (CRF) has been shown to induce various behavioral changes related to adaptation to stress. Dysregulation of the CRF system at any point can lead to a variety of psychiatric disorders, including substance use disorders (SUDs). CRF has been associated with stress-induced drug reinforcement. Extensive literature has identified CRF to play an important role in the molecular mechanisms that lead to an increase in susceptibility that precipitates relapse to SUDs. The CRF system has a heterogeneous role in SUDs. It enhances the acute effects of drugs of abuse and is also responsible for the potentiation of drug-induced neuroplasticity evoked during the withdrawal period. We present in this review the brain regions and circuitries where CRF is expressed and may participate in stress-induced drug abuse. Finally, we attempt to evaluate the role of modulating the CRF system as a possible therapeutic strategy for treating the dysregulation of emotional behaviors that result from the acute positive reinforcement of substances of abuse as well as the negative reinforcement produced by withdrawal.

Keywords: neuroplasticity, addiction, corticotropin releasing factor system, ethanol, anxiety, stress-induced

INTRODUCTION

Drug addiction is a chronic condition characterized by periods of abstinence and relapse. The effects of drugs of abuse on brain function have been extensively evaluated with the intention of developing therapies that can prevent relapse and facilitate the treatment of substance use disorders (SUDs). An extensive literature has shown that addictive drugs affect systems that govern reward pathways (mesolimbic dopaminergic pathway), learning and memory processes (hippocampus), emotion (amygdala), and cognitive functions (prefrontal cortex). The reinforcing effects of drug of abuse have been attributed to actions in the limbic system that in turn influence motivational, emotional and affective behaviors (Rezayof et al., 2002; David et al., 2008; Martin et al., 2008; Nielsen et al., 2011; Xue et al., 2012) and for reviews see (Koob, 1992; Pierce and Kumaresan, 2006; Feltenstein and See, 2008). Specifically, the alteration of reward processing (Wise, 1998, 2005) has been identified as a critical factor that leads to an increase in the chance of relapse (Koob and Le Moal, 1997; Everitt et al., 1999; Koob et al., 2004; Everitt and Robbins, 2005). The development of SUDs is a progression that commences with the first exposure to the drug and ends with physiological and psychological dependence.

Although substances of abuse have different mechanisms of action, repeated exposure has been shown to lead to similar neural adaptations. Addiction to any class of drugs has been described as a learning process. Individuals learn associations between the rewarding effects of the drugs and the environmental cues that predict drug availability. Neuroadaptations in areas associated with learning and memory (hippocampus and amygdala) are affected after a single episode of any drug use by influencing synaptic transmission. Following chronic drug use, the compulsive seeking and uncontrollable use leads to long-lasting alterations in synaptic plasticity, such as changes in synaptic strength.

Human studies (Gawin and Kleber, 1986; Wallace, 1989) and experiments with preclinical models (Thatcher-Britton and Koob, 1986; Piazza et al., 1990; Goeders and Guerin, 1994; Kreibich et al., 2009) have identified stress as a critical factor in the drug addiction process, including triggering relapse. Corticotropin releasing factor (CRF) has been implicated in neuroendocrine and behavioral responses to stress (Britton et al., 1982; Koob and Bloom, 1985). It has been shown to be activated during stress-induced drug reinstatement, where it acts to facilitate relapse and increase anxiety during acute and chronic withdrawal (Shaham et al., 1995; Ambrosio et al., 1997; Koob, 1999) and see (Sarnyai et al., 2001; George et al., 2011) for extensive review.

CRF-induced neuroplastic changes have been studied both in mesolimbic brain circuits that include the ventral tegmental area (VTA) and nucleus accumbens (NAcc) (Ungless et al., 2003; Wang et al., 2007a; Hahn et al., 2009) and also in brain regions associated with emotion, such as the amygdala (Fudge and Emiliano, 2003; Pollandt et al., 2006; Fu et al., 2007; Kash et al., 2008; Francesconi et al., 2009). Despite extensive research supporting the role of CRF in drug addiction, the specific participation of CRF on drug-induced synaptic plasticity that leads to relapse remains undetermined.

This review will attempt to examine recent research on the role of CRF and its interaction with drug-mediated synaptic plasticity. The VTA and the amygdalar nuclei where CRF is highly expressed will be described. We will discuss whether CRF

facilitates or inhibits synaptic strength from the basal condition. Finally, we will attempt to integrate the neurobiological changes that result from the interaction of substances of abuse with stress to evaluate alternative drug targets for experimental therapeutics to prevent relapse and facilitate the treatment of SUDs.

SUBSTANCE USE DISORDERS (SUDs) AND STRESS

SUDs are a chronic and relapsing condition characterized by an intense desire for drug intake during the withdrawal period. This craving process leads to a progression from the initial impulsive consumption to a subsequent compulsive and habit forming consumption that result in loss of control in limiting intake and subsequent inability to change the habit developed over time. One of the main challenges in preclinical addiction research has been to elucidate the pathways that lead to the loss of control of drug use and the predisposition to relapse (Koob and Le Moal, 1997). As described by the *Opponent Process Model,* the repetitive use of addictive substances alters the reward circuits by decreasing the intense pleasure state and by increasing the following unpleasant state. After discontinuation of repeated exposure to addictive drugs, compensatory reactions develop that oppose the primary effects of the drug—the withdrawal symptoms. The reduction of the withdrawal symptoms would therefore represent negative reinforcement. The reduction of the unpleasant state of the withdrawal symptoms becomes the major drive in continued drug use. In a simplified view of the *dopamine theory* (Wise, 1978, 2008; Berridge and Robinson, 1998; Everitt and Robbins, 2005; Diana, 2011), the acute euphoric process obtained by binge-intoxication represents the activation of the dopaminergic system, while the negative component resulting from the withdrawal period is marked by the reduction of dopamine function (Tomkins and Sellers, 2001). The introduction of *functional toxicity* (Weiss and Koob, 2001), which is associated with the unpleasant withdrawal state powered by the recruitment of the stress neurotransmitter, CRF, further expanded the *dopamine theory* as it applies to addiction.

CORTICOTROPIN RELEASING FACTOR (CRF) SYSTEM

CRF, also known as corticotropin releasing hormone (CRH), has been shown to induce various behavioral changes related to adaptation to stress. Dysregulation of the CRF system at any point can lead to a variety of psychiatric disorders such as depression, obsessive compulsive disorder, post-traumatic stress disorder and SUDs (Cole et al., 1990; Sarnyai et al., 1992, 2001; Cador et al., 1993; Koob and Kreek, 2007; Koob and Le Moal, 2008a). Footshock-induced stress has been shown to be effective in inducing reinstatement of alcohol (Le et al., 1998, 2000; Gass and Olive, 2007; Richards et al., 2008), nicotine (Buczek et al., 1999), cocaine (Erb et al., 1996), opiate and psycostimulants (Lu et al., 2003) and heroin (Shaham et al., 1997) seeking. Specifically CRF has been associated with drug reinstatement (Shaham et al., 1997; Le et al., 2002; Liu and Weiss, 2002; Funk et al., 2006). CRF has also been shown to produce anxiety-like behaviors during withdrawal from chronic ethanol (Baldwin et al., 1991; Overstreet et al., 2004) and may be responsible for persistent vulnerability and eventual relapse.

The CRF system consists of four ligands: CRF, urocortin (UCN) (Vaughan et al., 1995) 1, 2, and 3, two G-protein-coupled receptors (GPCR), CRF-receptor 1 (CRF-R1) and CRF-receptor 2 (CRF-R2), as well as a secreted CRF binding protein (CRF-BP); see **Table 1** and (Bale and Vale, 2004) for CRF system review.

It was originally identified as a hypothalamic factor responsible for stimulating adrenocorticotropic hormone (ACTH) secretion from the anterior pituitary (Guillemin and Rosenberg, 1955; Saffran et al., 1955) where it stimulates glucocorticoid synthesis and secretion form the adrenal cortex (Turnbull and Rivier, 1997). Its name was established thirty years before its biochemical identification in the 1980's (Vale et al., 1981) while its gene identifier in the National Center for Biotechnology Information (NCBI) is CRH. It is a 4.7-kilo-Dalton (kDa) peptide and consists of 41-amino acid residues. Neurosecretory neurons of the paraventricular nucleus (PVN) of the hypothalamus synthesize CRF (Meloni et al., 2005). CRF is then released into the afferent portal blood vessels to the anterior pituitary gland where it induces ACTH release in the systemic circulation. The hypothalamic-pituitary-adrenal (HPA) axis is regulated by negative feedback from glucocorticoids that activate glucocorticoid receptors specifically in the PVN and hippocampus. CRF is also expressed outside the HPA axis to control autonomic and behavioral responses to stressors (Palkovits et al., 1983; Swanson et al., 1983) including stress-induced reinstatement of drug seeking.

CRF mediates physiological stress responses by activating CRF-R1 and CRF-R2, which are distributed throughout the periphery and the brain (De Souza, 1995; Bale and Vale, 2004). It is believed that the binding of CRF to CRF-Rs is a two-step mechanism. The N-terminus of the receptor initially binds to the C-terminus of CRF, which initiates a rearrangement of the receptor (Grace et al., 2007). The CRF N-terminus contacts the other sites on the receptor to initiate cellular signaling (Vale et al., 1981; Rivier et al., 1984) and consequently activate the G-protein (Nielsen et al., 2000; Grace et al., 2004; Rijkers et al., 2004; Yamada et al., 2004; Hoare, 2005). The CRF system comprises other peptides with structural homology to CRF. UCN 1 shows 45% sequence identity with CRF and binds with high affinity to both CRF receptor subtypes (Perrin et al., 1995), whereas CRF binds with highest affinity to CRF-R1 (Vaughan et al., 1995; Burnett, 2005). UCN 2, also known as stresscopin related peptide, and UCN 3, also known as stresscopin bind specifically to CRF-R2 (Hsu and Hsueh, 2001; Lewis et al., 2001; Reyes et al., 2001).

CRF-R1 has 415 amino acid residues and it is expressed in the periphery and in the CNS (Chang et al., 1993; Chen et al., 1993; Vita et al., 1993; Potter et al., 1994; Tsai-Morris et al., 1996; Sanchez et al., 1999; Van Pett et al., 2000). Chronic stress mediated by activation of CRF-R1 by CRF has been associated with the development of anxiety disorders (Arborelius et al., 1999); CRF-R1 antagonists have been shown to reduce anxiety-like behaviors (Funk et al., 2007). Transgenic mice with deletion of CRF-R1 (CRF-R1 knock out (KO) mice) have reduced reaction to both stress and anxiety, for comprehensive review see (Bale and Vale, 2004). This anxiolytic effect, however may be attributed to the reduction in circulating glucocorticoids in preclinical models (Tronche et al., 1999).

Table 1 | Corticotropin Releasing Factor (CRF) system.

Name	Type	Receptor binding	CNS expression	Peripheral expression	Involvement in stress response
CRF	ligand	CRF-R1 > CRF-R2	synthesized in PVN widely distributed	gut, skin, adrenal gland	HPA axis: induces ACTH release outside HPA axis: controls autonomic and behavioral responses
CRF-R1	receptor	–	CC, CB, MS, HIP, VTA, amygdala, pituitary	β cell pancreas	anxiogenic
CRF-R2	receptor	–	RN, LS, HY, CP	heart, GI, lung, skeletal muscle, vasculature	anxiogenic/anxiolytic
CRF-BP	binding protein	–	CC, HY, amygdala, VTA	Plasma, amniotic fluid, placenta, pituitary gland, liver	Periphery: neutralizes CRF CNS: undetermined
UCN 1	ligand	CRF-R1/CRF-R2	EW	GI, testis, cardiac myocytes, thymus, skin, spleen	Periphery: elevated in heart failure (Wright et al., 2009) CNS: modulate excitatory glutamatergic synaptic transmission (Liu et al., 2004)
UCN 2	ligand	CRF-R2	HY, brainstem, spinal cord	heart, blood cells, adrenal gland	central autonomic and appetitive control (Reyes et al., 2001) gender difference in depressive-like behavior (Chen et al., 2006)
UCN 3	ligand	CRF-R2	HY, amygdala	GI, pancreas	energy homeostasis (Li et al., 2007) anxiolytic-like effects (Valdez et al., 2003)

CeA, central nucleus of the amygdala; CB, cerebellum; CC, cerebral cortex; CP, choroid plexus; EW, cell bodies of the Edinger Westphal nucleus; GI, gastrointestinal tract; HIP, hippocampus; HY, hypothalamus; LS, lateral septum; MS, medial septum; OLF, olfactory area; PVN, paraventricular nucleus of the hypothalamus; RN, raphe nuclei.

A conditional KO mouse line was generated to differentiate the behavioral from the neuroendocrine CRF-mediated CRF-R1 signaling pathways. The selective inactivation of the limbic structures, but not of the HPA system has shown that CRF-R1 modulates anxiety-like behaviors and it is independent of the HPA (Muller et al., 2003). Furthermore, CRF-R1 is thought to increase susceptibility to alcohol relapse behaviors (Hansson et al., 2006; Heilig and Koob, 2007). A recent study evaluated the role of CRF both within and outside the HPA has shown that CRF via CRF-R1 signaling may have opposite effects on stress-related alcohol consumption (Molander et al., 2012).

CRF-R2 has three variants: α, β, and γ. The α is comprised of 411 amino acid residues and the β is comprised of 413–418 amino acid residues. Both are found in the brain and periphery; however, CRF-R2β is predominantly found in the heart and vasculature (Lovenberg et al., 1995a,b; Kimura et al., 2002; Burnett, 2005). The γ variant is a smaller peptide containing only 397 amino acid residues, and is found only in the human brain (Kostich et al., 1998). The precise role of CRF-R2 in the regulation of the stress response is a subject of intense investigation. Genetic mouse model studies with deletion of CRF-R2 (CRF-R2 KO mice) have demonstrated that CRF activation of CRF-R2 can lead to either an increased or decreased response to stressors (Bale et al., 2000, 2002; Coste et al., 2000; Kishimoto et al., 2000).

The lack of specific antisera that support immunohistochemical experiments and the low resolution of ligand binding approaches have limited the studies to elucidate the CRF-Rs distribution and limit the analysis at the mRNA level. To overcome this impediment, a transgenic mouse that reports expression of CRF-R1 with green fluorescent protein (GFP) has been successfully generated providing a novel tool to investigate the role of CRF-R1 signaling in stress adaptation (Justice et al., 2008).

CRF-BP is a water-soluble, 37 kD protein and consists of 322 amino acid residues (Bale and Vale, 2004). It is a secreted glycoprotein, efficiently stored into secretory granules and released into the extracellular space through exocytosis (Blanco et al., 2011). It contains aspargine N-linked-type oligosaccharides that are critical for CRF-BP binding to CRF (Suda et al., 1989). Previous attempts to identify small molecule inhibitors of CRF-BP have produced limited success due in part to the high affinity (picomolar) of CRF binding to CRF-BP (Behan et al., 1995a) and also because CRF-BP full length (FL) is susceptible to autocatalytic proteolysis (Woods et al., 1999). The spontaneous proteolytic

cleavage yields a larger N-terminal fragment of 27 kD, CRF-BP (27 kD), which retains the binding site for CRF and a smaller, 9.6 kD C-terminal fragment, CRF-BP (10 kD) (Woods et al., 1999) with no apparent physiological or pathological role. The unique cleavage site in CRF-BP (FL) has been identified between amino acid residues serine 234 and alanine 235. The generation of two fragments has made it extremely difficult to successfully purify sufficient quantities of CRF-BP (FL) to study the physiological properties of the native protein. CRF-BP is distributed in plasma, amniotic and synovial fluid, the placenta, the pituitary gland, the liver, and in several distinct brain regions, including the cerebral cortex, the hippocampus (Behan et al., 1995a), the amygdala (Herringa et al., 2004) and the VTA (Wang and Morales, 2008). In the periphery, circulating CRF-BP neutralizes the physiological actions of CRF (Kemp et al., 1998). Because of the high affinity with CRF, it is believed that CRF-BP plays a buffer role by reducing the amount of free CRF. In the brain, however, CRF-BP is mostly membrane-bound and expressed in different amounts in neurons and neuroglial cells (Behan et al., 1995b). Within neuronal cells, recent findings demonstrated that discrete subpopulations of VTA dopaminergic and γ-aminobutyric acid (GABAergic) neurons express CRF-BP (Wang and Morales, 2008). The physiological role of CRF-BP in the central nervous system (CNS) is still unclear. Additionally, theories suggest the possibility that CRF-BP may assist the clearance of CRF from the body and may also protect CRF from degradation (Seasholtz et al., 2002). Genetic mouse model studies with deletion of CRF-BP (CRF-BP KO mice) have shown there is an increase in anxiety-like behavior (Karolyi et al., 1999). Electrophysiology studies have shown that CRF signals through CRF-R2 to potentiate N-Methyl-D-aspartate (NMDA)-mediated excitatory postsynaptic currents (EPSCs) in the VTA (Ungless et al., 2003). Furthermore, using CRF (6–33), a peptide that competes with CRF at the CRF-BP binding site, but does not bind to CRF-R2, it was shown that it blocked CRF-induced potentiation of NMDAR-mediated EPSCs (Ungless et al., 2003). Taken together, these results suggest that CRF-BP possesses a diverse role in modulating the CRF-system. As described by *in vitro* and *in vivo* studies, purifying human CRF-BP (FL) in sufficient quantities for investigation has not been successful to date (Woods et al., 1997). There have not been any research tools available to characterize the role of CRF-BP in the CNS by expressing CRF-BP on the cell surface. Therefore, it has not been possible to determine whether CRF-BP participates specifically in the CRF-R2 signaling. A summary of the involvement of the CRF binding in addictive behavior is described in **Table 2**.

Table 2 | Involvement of the CRF binding in addictive behaviors.

CRF-R1 antagonists	Attenuate stress-induced relapse to drug seeking and behavioral changes associated with withdrawal; small molecules and peptides are available for investigation
CRF-R2 antagonists	Regulation of the stress response and addictive behavior is unclear; small molecules and peptides are available for investigation
CRF-BP antagonists	Modulation of neuronal activity may be a target for both drugs of abuse and stress response; only peptides are available for investigation

STRESS-INDUCED DRUG ADDICTION: CRF-MEDIATED NEUROTRANSMISSION AND PLASTICITY

REINFORCEMENT: VENTRAL TEGMENTAL AREA (VTA) AND NUCLEUS ACCUMBENS (NAcc)

Addictive drugs have been shown to increase the concentration of dopamine in the NAcc. Furthermore, the increase of dopamine has been associated with the amplification of the hedonic impact of positive reinforcers (Fibiger, 1978; Berridge et al., 1989) and the development of addictive behaviors (Yokel and Wise, 1975; Bonci and Malenka, 1999; Wise, 2008). The NAcc receives input from the VTA and it is thought that this pathway may be responsible not only for the acute pleasure effect of drug intake, but also for the negative reinforcement and the effects of cues on drug-seeking behaviors (Koob and Nestler, 1997).

CRF cellular involvement in the VTA

The VTA receives CRF projections mostly from the limbic forebrain and PVN of the hypothalamus (Rodaros et al., 2007) that form glutamatergic synapses and symmetric GABAergic synapses (Tagliaferro and Morales, 2008). The PVN is the site for CRF synthesis (Meloni et al., 2005) and the majority of asymmetric synapses (glutamatergic) are expressed in CRF- and dopaminergic-containing neurons. VTA dopaminergic neurons express CRF-R1 (Van Pett et al., 2000) and a more recent study has shown that the majority of VTA neurons expressing CRF-BP are dopaminergic (Wang and Morales, 2008). The CRF system modulates dopaminergic neurons by activating CRF-R1 and CRF-R2; however, CRF is not only involved in the neuroexcitability of the dopaminergic system. It may also be responsible for modulating excitatory and inhibitory synaptic inputs since the VTA receive inputs from both CRF-glutamatergic- and CRF-GABAergic-containing neurons (Tagliaferro and Morales, 2008) and for review see Borgland et al. (2010).

CRF increases the firing rate of VTA dopaminergic neurons (Korotkova et al., 2006; Wanat et al., 2008) via CRF-R1, and involves the phospholipase C (PLC)–protein kinase C (PKC) signaling pathway with enhancement of I_h (hyperpolarization-activated inward current) (Wanat et al., 2008). CRF can also induce a transient slowly developing potentiation of NMDA-mediated synaptic transmission via CRF-R2 and activation of the PLC-PKC signaling pathway. CRF-R2-mediated potentiation has been shown to require the presence of CRF-BP (Ungless et al., 2003). The mechanism of action of CRF-R2 and CRF-BP is still under investigation as the research tools needed to study CRF-BP and antisera that specifically target CRF-R2 have not been available.

CRF appears to have both excitatory and inhibitory actions on the dopaminergic neurons in the VTA. Studies using cocaine and methamphetamine have shown that the excitatory effect of CRF on dopaminergic neurons involves fast events, for example action potential firing rate and NMDAR-mediated synaptic

transmission, while the inhibitory effects of CRF involve slow forms of synaptic transmission that would result in long-term plasticity (Beckstead et al., 2009). Those observations demonstrated that CRF may have different actions on receptors that mediate the synaptic action on dopamine. This cellular mechanism may refine the role of stress by CRF actions on dopamine-mediated behaviors (Beckstead et al., 2009).

As it has been shown that potentiation of CRF-R2, but not CRF-R1, signaling requires the presence of CRF-BP (Ungless et al., 2003), it has been proposed that CRF-BP and CRF-R2 mediate longer-lasting forms of synaptic plasticity (Bonci and Malenka, 1999). Both behavioral sensitization and long-term potentiation (LTP) share many characteristics such as the involvement of NMDAR activation for the induction of LTP in VTA dopaminergic neurons (Bonci and Malenka, 1999; Ungless et al., 2001). As a consequence, it has been suggested that synaptic plasticity at excitatory synapses on VTA dopaminergic neurons may play a principal role in triggering behavioral change. Since NMDAR activation is required for the induction of LTP in VTA dopaminergic neurons, CRF-Rs activation may modulate longer-lasting forms of plasticity (Bonci and Malenka, 1999; Ungless et al., 2001; Bonci and Borgland, 2009).

CRF-mediated neurotransmission and plasticity

Synaptic adaptations observed in remodeling of neuronal circuits in addictive drug studies have been shown to have implications in behavior and memory traits that characterize SUDs. The neuroplasticity underlying drug-induced sensitization has produced a growing body of evidence that suggests it may represent the molecular effect that is critical in modulating addictive behaviors and would contribute to stress-induced compulsive behaviors in addiction.

Axon terminals of CRF neurons synapse onto VTA neuronal dendrites (Tagliaferro and Morales, 2008) and it appears that stress affects the CRF release in this region (Wang et al., 2006). Electrophysiological studies have shown that CRF-BP is required for a slowly developing, transient potentiation of NMDAR-mediated synaptic transmission elicited by CRF via CRF-R2 specifically (Ungless et al., 2003). These results have been corroborated by behavioral studies that determined the effectiveness of stress in triggering glutamate and dopamine release in cocaine seeking of drug-experienced rats (Wang et al., 2007b). Using chronic cocaine preclinical models, the study has shown the positive reinforcement associated with CRF, specifically CRF/CRF-R2/CRF-BP interaction with the dopaminergic system. Those findings support additional research efforts to develop novel approaches that probe CRF-BP on the cell surface.

In conclusion, CRF increases VTA glutamatergic synaptic function, which may facilitate VTA burst firing or induction of synaptic plasticity that may result from repeated exposure to drugs of abuse. This process may produce long-term neuroadaptations that alter stress responses and enhance drug seeking. Electrophysiological studies combined with behavioral studies have proposed that previous experience with drugs of abuse may facilitate the ability of stress to drive drug seeking and, therefore, relapse. These results suggest that CRF may be important for drug-evoked synaptic plasticity in VTA dopaminergic neurons and may represent the molecular substrate that explains the anxiety and stress response during withdrawal from substances of abuse.

CELLULAR INVOLVEMENT OF CRF IN THE AMYGDALA

The amygdala is believed to be a pivotal brain region for emotional response and it is critical for providing affective salience to sensory information (Adolphs et al., 1994; LeDoux, 2003; Phelps and LeDoux, 2005). Negative affective responses have been studied in specific nuclei of the amygdala by studying the conditioned fear response (Davis, 1992a,b). The amygdala is widely connected to other limbic regions where it participates in integrating sensory and cognitive information (LeDoux, 1992, 1993). Experimental evidence strongly suggests drugs of abuse act on this system and can modify synaptic events especially during withdrawal. While the VTA has been associated with the reinforcing effects of ethanol (Gatto et al., 1994), the activation of the GABAergic system has been associated with alcohol's anxiolytic effect (Frye and Breese, 1982). In addition to the rewarding circuits of the shell of the NAcc, and brain regions activated by pharmacological stressor, such as yohimbine and footshock were found to be specific in the basolateral and central amygdalar nuclei, and the bed nucleus of the stria terminalis (BNST) (Funk et al., 2006). Preclinical studies demonstrated that exposure and withdrawal from ethanol induces functional and biochemical changes in the amygdala of rats, demonstrating that this circuit is involved in long-term increases in anxiety-like behavior following chronic ethanol exposure (Christian et al., 2012).

The amygdala mediates conditioned and unconditioned responses to aversive stimuli (Davis and Whalen, 2001) and it has been investigated using Pavlovian fear conditioning by pairing a conditioned stimulus with an aversive unconditioned stimulus. The re-exposure of the unconditioned stimulus elicits a conditioned fear response derived by the conditioned-unconditioned association (Pitts et al., 2009). The association signal takes place in the basolateral amygdala (BLA) and is then transmitted to the central nucleus of the amygdala (CeA) (McDonald, 1998; Maren, 1999; Davis and Shi, 2000; Pitkanen et al., 2000; Pare et al., 2004). This transmission process involves both positive and negative associations.

All components of the CRF system, CRF, CRF-Rs and CRF-BP are expressed in the amygdala (Potter et al., 1994). Furthermore, the amygdala is a major extrahypothalamic source of CRF-containing neurons (Palkovits et al., 1983; Van Pett et al., 2000). Both BLA and CeA nuclei play a role in the stress response (Richter et al., 1995; Merali et al., 1998; Koob and Heinrichs, 1999). Extensive studies have shown that the CRF system participates in memory consolidation that involves the BLA-CeA circuit (Roozendaal et al., 2002; Hubbard et al., 2007). It has been observed that CRF release in the amygdala is increased during acute withdrawal (Richter and Weiss, 1999); therefore, it has been hypothesized that CRF may modulate drug-evoked synaptic plasticity (Ungless et al., 2001, 2003) and for a recent review, see (Luscher and Malenka, 2011). The neuronal basis for negative reinforcement is less well-understood; however, more recent behavioral studies have shown that CRF is capable of

potentiating excitatory synaptic currents via CRF-R1 in the CeA two weeks following withdrawal from cocaine (Pollandt et al., 2006).

A recent study has shown that CRF-R1 specifically possess a bidirectional role in anxiety (Refojo et al., 2011). While deletion of CRF-R1 in the mid brain dopaminergic neurons increases anxiety-like behaviors and reduces dopamine release in the prefrontal cortex, deletion of CRF-R1 in the forebrain glutamanergic neuronal network reduces anxiety and disrupts transmission in the amygdala and hippocampus (Refojo et al., 2011).

The role of CRF was also evaluated extensively in voluntary ethanol consumption using gene expression and genetic variation in preclinical models see (Bjork et al., 2010) for extensive review. In ethanol-exposed animals, ethanol intake was reduced by administration of CRF-R1 antagonist, and tested using pharmacological interventions that reduce anxiety-like behaviors (Logrip et al., 2011; Zorrilla and Koob, 2012). The reduction of ethanol intake was also observed in transgenic mice with deletion of CRF-R1 (CRF-R1 KO) (Chu et al., 2007). CRF-R1 antagonists reduce drug withdrawal-associated anxiety and attenuate the negative reinforcing effects of ethanol associated with prolonged ethanol exposure (Ghitza et al., 2006; Marinelli et al., 2007; Li et al., 2007; Koob and Le Moal, 2008b; Richards et al., 2008). CRF-R1 inhibitors have shown to attenuate stress-induced relapse to cocaine and heroin in trained animals (Shaham et al., 1998) and to reduce stress-induced reinstatement and stress-induced reactivation of conditioned place preference in many addictive drugs (Koob and Zorrilla, 2010).

The extended amygdala

Among the extrahypothalamic structures that contain CRF expressing neurons there is the "extended amygdala." The extended amygdala is comprised by the BNST, the central medial amygdala (CeA), the sublenticular sustantia innominata and a transition zone forming the posterior part of the NAcc (Heimer and Alheid, 1991). It represents the brain circuit involved in processing the aversive stimuli produced by ethanol withdrawal (Koob and Le Moal, 2001), in which the GABA system has been altered and the CRF system in the adjacent CeA has been shown to be activated (Roberts et al., 1996). Those observations indicate that GABAergic activity within interneurons of the extended amygdala may play a prominent role in the chronic negative emotion-like state of motivational significance for drug seeking in alcohol dependence (Koob and Le Moal, 2001; Koob, 2003, 2009a,b). In addition, an *in situ* hybridization study has shown that recruitment of CRF-R1 signaling, in the components of the extended amygdala, may be responsible of driving the excessive voluntary alcohol intake and may be linked to increase stress activity (Hansson et al., 2007).

The BNST (as well as distinct regions of the CeA) has been associated with stress and anxiety (Walker and Davis, 2008) and is involved specifically with CRF signaling (Davis et al., 1997). The CeA and BNST have direct projections to many brain regions that have been studied to elucidate the symptoms of fear or anxiety (Davis, 1992b). The BNST has been identified as a possible regulator of VTA dopaminergic neuron firing (Georges and Aston-Jones, 2002) and consequently involved in the regulation of acute actions of alcohol, nicotine, and cocaine (Watkins et al., 1999; Carboni et al., 2000; Eiler et al., 2003).

The BNST possesses an extensive network of dopaminergic fibers (Fudge and Emiliano, 2003) and is connected to the reward pathway by extensive projections to the VTA, thus influencing the excitatory input through both NMDA and non-NMDA receptors (Georges and Aston-Jones, 2001, 2002). This dopaminergic excitatory transmission in the VTA requires the presence of CRF (Kash et al., 2008). Acute cocaine administration has been shown to induce dopamine signaling through a specific CRF-R1-dependent enhancement of NMDA excitatory transmission (Kash et al., 2008). This mechanism was described as a short-term form of plasticity in the BNST, which may be responsible for the acute effects of addictive drugs (Kash et al., 2008). These findings suggested that glutamatergic neurotransmission in BNST may be functionally involved with acute reinforcing actions of drug of abuse (Walker and Davis, 2008).

Basolateral amygdala (BLA)

The basolateral nucleus of the amygdala (BLA) is critically implicated in emotional learning (LeDoux, 2000), and in reward (Balleine and Killcross, 2006; Tye et al., 2008). Neurons from the BLA project directly to the CeA as well as to the BNST. The BLA is mostly composed by glutamatergic pyramidal neurons and provides the main excitatory input to the CeA and other limbic and cortical structures (Sah et al., 2003); however, the excitatory transmission is believed to be modulated by the relatively small number of GABAergic interneurons found there (Washburn and Moises, 1992). GABAergic interneurons have been identified as regulators of stress and anxiety (Silberman et al., 2009).

CRF is present abundantly in the BLA, in addition to CRF-R1 and CRF-BP, (Sakanaka et al., 1986; Potter et al., 1992; Van Pett et al., 2000); however, the effects of CRF in the BLA have been studied far less than the other nuclei of the amygdala. The BLA has been shown to be a critical nucleus for the consolidation of fear and memory and, therefore, is a possible target for dampening emotional memories. It has been shown that intra BLA infusions of CRF increase anxiety-like behaviors (anorexia and grooming) that are blocked by the administration of a CRF-R1 antagonist (Jochman et al., 2005). Another BLA microinfusion study showed that CRF-R1 activates fear memory consolidation and that this effect is blocked by administration of another CRF-R1 antagonist. The fear memory consolidation process seems specifically regulated by the CRF-R1 activation since CRF-R2 antagonist in the BLA disrupted neither the contextual fear conditioning nor performance of contextual freezing in the drug-free conditioned fear test (Hubbard et al., 2007). BLA CRF-R1 activation has been described as induced synaptic plasticity, and demonstrating that BLA CRF-R1 activation can be pharmacologically blocked by small molecules, the possibility to compromise the consolidation of fear memory suggests a potential therapeutic opportunity to ease the development of intense emotional memories.

Central nucleus amygdala (CeA)

The CeA has been identified as locus for both acute positive reinforcement of ethanol self-administration and for the negative reinforcement associated with ethanol withdrawal (Baldwin

et al., 1991; Heinrichs et al., 1992, 1995; Koob and Le Moal, 1997, 2001; Zorrilla et al., 2001). The CeA has also been identified as a critical locus for reversing many behavioral effects associated with ethanol intoxication (Hyytia and Koob, 1995).

In the CeA, most neurons are GABAergic (Sun and Cassell, 1993), and CRF is highly co-expressed with GABAergic neurons (Veinante et al., 1997; Day et al., 1999). The CeA abundantly expresses CRF, CRF-R1 and CRF-BP (Sakanaka et al., 1986; Potter et al., 1992; Van Pett et al., 2000). Moreover, in the CeA the action of CRF and ethanol has been shown to increase GABA release (Nie et al., 2004) and the amount of CRF release is increased in preclinical models of ethanol dependence (Merlo Pich et al., 1995). Protein kinase C epsilon (PKCε) has been shown to modulate CRF-R1 signaling in the CeA (Choi et al., 2002) and transgenic mice with deletion of PKCε (PKCε KO mice) have shown reduced anxiety-like behaviors (Hodge et al., 2002). Electrophysiological studies have shown that ethanol-induced GABA release in the amygdala is regulated by CRF-R1 (Nie et al., 2004) and that ethanol-stimulated vesicular GABA release depends on PKCε models (Bajo et al., 2008). PKCε signaling pathway in the CeA is activated by CRF-R1 activation and modulates GABAergic neurotransmission that may contribute to the anxiogenic effects of ethanol (Smith et al., 1998; Timpl et al., 1998). This functional link between ethanol, CRF and PKCε that modulates GABAergic neurotransmission in the CeA may contribute to the dysregulation of emotional behaviors that regulate acute positive reinforcement of ethanol consumption and the negative reinforcement produced by ethanol withdrawal.

It has been shown that there is a critical difference between CRF effects in low/moderate ethanol-exposed animals (binge-like ethanol consumption) and ethanol-dependent animals (chronic-like ethanol exposure). While binge-like ethanol (Lowery-Gionta et al., 2012) may cause transient perturbations of the CRF system which may be able to return to its *homeostatic state,* the chronic-like ethanol exposure (Roberto et al., 2003, 2004) may be responsible for the CRF neuroadaptation that would influence the *allostatic state*. An *allostatic state* is defined as a state of chronic deviation of the regulatory network from their normal process and the establishment of a different set point of "apparent stability" (Koob and Le Moal, 2001). This chronic deviation of reward set point is critically altered during drug withdrawal and may contribute to subsequent neuroadaptation that produces vulnerability to addiction and relapses (Koob and Le Moal, 2001). Acute stress does not increase the mRNA expression of any components of the CRF system in the CeA (Herringa et al., 2004), however, in the CeA of animals exposed to ethanol, there was a significant increase in CRF mRNA expression (Lack et al., 2005) as well as in ethanol-dependent animals during withdrawal (Sommer et al., 2008).

The recruitment of CRF in the CeA during early drinking episodes, before dependence, may initiate neuroplastic changes in the system that may become more intense with additional ethanol exposures (Lowery-Gionta et al., 2012). It has been proposed that this CRF-dependent change contributes to the transition from binge-drinking to ethanol dependence (Lowery-Gionta et al., 2012). The authors also found that ethanol enhances GABAergic transmission in the amygdala at both pre- and post-synaptic sites in ethanol naïve animals, while binge ethanol consumption blunts the CRF-mediated GABAergic transmission (Lowery-Gionta et al., 2012). This study revealed that drinking reduced the effect CRF has on GABAergic transmission. In contrast, others have found that animals dependent on ethanol showed enhanced GABAergic transmission in the CeA (Roberto et al., 2004).

CRF and norepinephrine have been shown to increase GABAergic activity measured by $GABA_A$ inhibitory postsynaptic potential (IPSCs) in whole-cell recording from the CeA. This effect was blocked by CRF-R1 antagonists and blocked in CRF-R1 knockout mice (Nie et al., 2004; Kash and Winder, 2006). The augmented GABA release produced by ethanol in the CeA in dependent animals was observed both in electrophysiological and *in vivo* microdialysis experiments (Roberto et al., 2003). Later studies in ethanol-dependent rats corroborated that CRF-alcohol interaction on GABAergic transmission in the CeA is more pronounced during alcohol dependence (Roberto et al., 2004).

CONCLUSIONS

This review has summarized the multiple mechanisms that underlie persistent changes in synaptic efficacy following administration of addictive drugs. It is evident that the CRF system significantly facilitates the induction and maintenance of plasticity in the VTA and amygdala, with resulting enhancement of glutamate-mediated excitation and reduction of GABA-mediated inhibition, thus contributing to the molecular basis of drug addiction.

Neuroplasticity in brain reward circuitry following a history of ethanol dependence has been shown (Hansson et al., 2008). Experimental data illustrated in this review support the hypothesis that stress induces plasticity within the VTA and amygdala nuclei and may participate in the development of a chronic anxiety state that could lead to the development of SUDs. These changes in the limbic neuronal network may represent the trigger that may lead to loss of control of drug use. Addictive drugs have been shown to induce behavioral sensitization and there is a large body of literature that evaluates the role of stress and addictive behaviors. Studies of long-term neuroadaptation in alcohol addiction have shown that brain stress and fear systems become activated (Heilig et al., 2010); however, there is still much to be elucidated pertaining to drugs' actions on the CRF system, both in regard to synaptic plasticity and behavioral responses. Several blood–brain barrier-penetrating CRF-R1 antagonists have been developed, however while some compounds have shown efficacy in animal models to treat alcoholism (Gehlert et al., 2007, 2012), CRF-R1 antagonists have still not succeeded in clinical trials (Koob and Zorrilla, 2012).

Preventing all exposure to substances of abuse is almost impossible, as many psychoactive substances (alcohol, nicotine, caffeine, and prescription medications) are generally accepted in our society. There are many medications that are FDA approved or used off-label for alcohol dependence that focus on the treatment of symptom reduction (disulfuram, naltrexone), assistance with withdrawal (benzodiazepines, valproic acid, varenicline), and relapse prevention (acamprosate, ondansetron, baclofen,

topiramate, varenicline, methadone) and others FDA approved medications for other indications are at the preclinical stage (mifepristone) (Simms et al., 2011), however, the recidivism in drug abuse is still a major problem for SUDs. Although different classes of substances of abuse have different mechanisms of action, repeated drug use leads to stimulation of the HPA axis and the abrupt cessation of chronic drug use increases activation of CRF. Medications that modulate stress responses may offer a novel pharmacotherapeutic approach for SUDs. Regulating stress outcomes by acting on the CRF system may offer the possibility to develop that novel therapeutic directed to diminish the effect of CRF in synaptic transmissions. By easing the stress-induced drug seeking, it may be possible to reduce relapse and facilitate the formation of memories with less deleterious behavioral consequences.

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Alcohol-induced plasticity in the dynorphin/kappa-opioid receptor system

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Alcoholism is a chronic relapsing disorder characterized by continued alcohol use despite numerous adverse consequences. Alcohol has been shown to interact with numerous neurotransmitter systems to exert its pharmacological effects. The endogenous opioid system (EOS) has been strongly implicated in the positive and negative reinforcing effects of alcohol. Traditionally recognized as dysphoric/anhedonic in nature, the dynorphin/kappa-opioid receptor (DYN/KOR) system has recently received considerable attention due to evidence suggesting that an upregulated DYN/KOR system may be a critical contributor to the complex factors that result in escalated alcohol consumption once dependent. The present review will discuss alcohol-induced plasticity in the DYN/KOR system and how these neuroadaptations could contribute to excessive alcohol seeking and consumption.

Keywords: alcohol, dependence, depression, dynorphin, ethanol, kappa-opioid receptor, negative affect, withdrawal

INTRODUCTION

Alcohol use disorders, comprising alcohol abuse and dependence, pose a substantial physical, mental, and fiscal health risk to millions of people each year in the US, causing an average of 79,000 deaths and costing $224 billion annually (Grant et al., 2004; Bouchery et al., 2011). Alcohol exerts it's effects through multiple neurotransmitter systems, e.g., dopamine (DA), glutamate, γ-aminobutyric acid (GABA), and serotonin (5-HT) systems (Colombo et al., 2004; Johnson, 2004; Walker and Ettenberg, 2007; Heinz et al., 2009). In particular, the endogenous opioid system (EOS) has proven to be important when considering the positive reinforcing effects of alcohol. Furthermore, the EOS undergoes neuroadaptations following chronic alcohol exposure that lay the foundation for alcohol to serve as a potent negative reinforcer during both acute and protracted withdrawal following chronic alcohol exposure (for a review of negative reinforcement from a learning perspective, see Walker, 2012).

Acute alcohol stimulates the release of β-endorphin (βEND), enkephalin (ENK), and dynorphin (DYN) (Gianoulakis et al., 1996; Marinelli et al., 2003, 2004, 2005, 2006; Dai et al., 2005; Lam et al., 2008; Jarjour et al., 2009). βEND and ENK, endogenous ligands for μ-(MOR) and δ-(DOR) opioid receptors, respectively, have been linked to euphoric and positive reinforcing effects of alcohol (Stromberg et al., 1998; Hyytia and Kiianmaa, 2001). Conversely, DYN, the endogenous ligand for the κ-opioid receptors (KORs) (Chavkin et al., 1982), has been shown to produce aversive effects related to alcohol challenge (Lindholm et al., 2001). There are two forms of DYN, DYN A, and DYN B, although for the purposes of this review, they will be collectively called DYN because the precise neurobehavioral differences between the two has yet to be established. The KOR is the preferential binding site for DYN (Chavkin et al., 1982; Merg et al., 2006), although DYN has affinity for all three opioid receptors (Merg et al., 2006; Schwarzer, 2009).

The KOR, a G-protein coupled receptor, induces inhibitory signaling (Connor and Christie, 1999; Al-Hasani and Bruchas, 2011; but see Crain and Shen, 1990) and has been shown to regulate the release of various neurotransmitters including DA, glutamate, GABA, norepinephrine (NE), and 5-HT (Mulder et al., 1984; Jackisch et al., 1986; Schoffelmeer et al., 1997; Shippenberg and Rea, 1997; Rawls et al., 1999; Shippenberg et al., 2007; Land et al., 2009). Details of these interactions have been reviewed

previously (Vengeliene et al., 2008; Heinz et al., 2009). The dynorphin/kappa-opioid receptor (DYN/KOR) system is widely distributed in the CNS and has been implicated in numerous physiological and pathophysiological conditions related to mood and motivation (Bruijnzeel, 2009; Schwarzer, 2009; Wee and Koob, 2010; Tejeda et al., 2012), identifying the DYN/KOR system as a putative therapeutic target for the treatment of various neuropsychiatric disorders (Walker and Koob, 2008; Knoll and Carlezon, 2010; Wee and Koob, 2010; Tejeda et al., 2012; Walker et al., 2012). Also becoming apparent, is the importance of the dysphoric/anhedonic properties of a hyperactive DYN/KOR system in alcohol dependence that contributes to the negative reinforcing effects of alcohol (Walker and Koob, 2008; Nealey et al., 2011; Walker et al., 2011). The present review focuses on alcohol-induced plasticity in the DYN/KOR system and how these neuroadaptations perpetuate excessive alcohol seeking and consumption.

THE DYN/KOR SYSTEM IN MOTIVATIONAL AND EMOTIONAL NEUROCIRCUITRY

Neuropharmacological studies have identified brain regions mediating the reinforcing effects of alcohol and other drugs of abuse. The mesolimbocortical dopamine system [DA from the ventral tegmental area (VTA) to the nucleus accumbens (Acb) or prefrontal cortex (PFC)] and extended amygdala [central nucleus of the amygdala (CeA), bed nucleus of the stria terminalis (BNST) and Acb Shell (AcbSh)] represent neurocircuitry related to motivational and cognitive decision making, as well as emotional, neurocircuitry thought to participate in the reinforcing effects of alcohol. **Figure 1** summarizes brain motivational and emotional neurocircuitry and illustrates several relevant neurotransmitter systems that can be modulated by KORs and are implicated in the pathophysiology of various psychiatric disorders following exposure to drugs of abuse, including alcohol. KORs are presynaptically positioned on dopaminergic inputs to amygdala (Amyg), Acb and PFC (Werling et al., 1988; Frankhuijzen et al., 1991; Grilli et al., 2009), GABAergic inputs to Acb, Amyg and BNST (Hjelmstad and Fields, 2003), serotonergic inputs to Acb (Land et al., 2009), noradrenergic inputs to PFC (Berger et al., 2006) and glutamatergic inputs to VTA and Acb (Hjelmstad and Fields, 2001). KORs are also found directly on the perikarya of dorsal raphe 5-HT neurons (Land et al., 2009), locus coeruleus NE neurons (Reyes et al., 2009) and VTA mesocortical DA neurons (Margolis et al., 2006). As such, KORs are positioned to modulate numerous neurotransmitter systems implicated in neuropsychiatric disorders

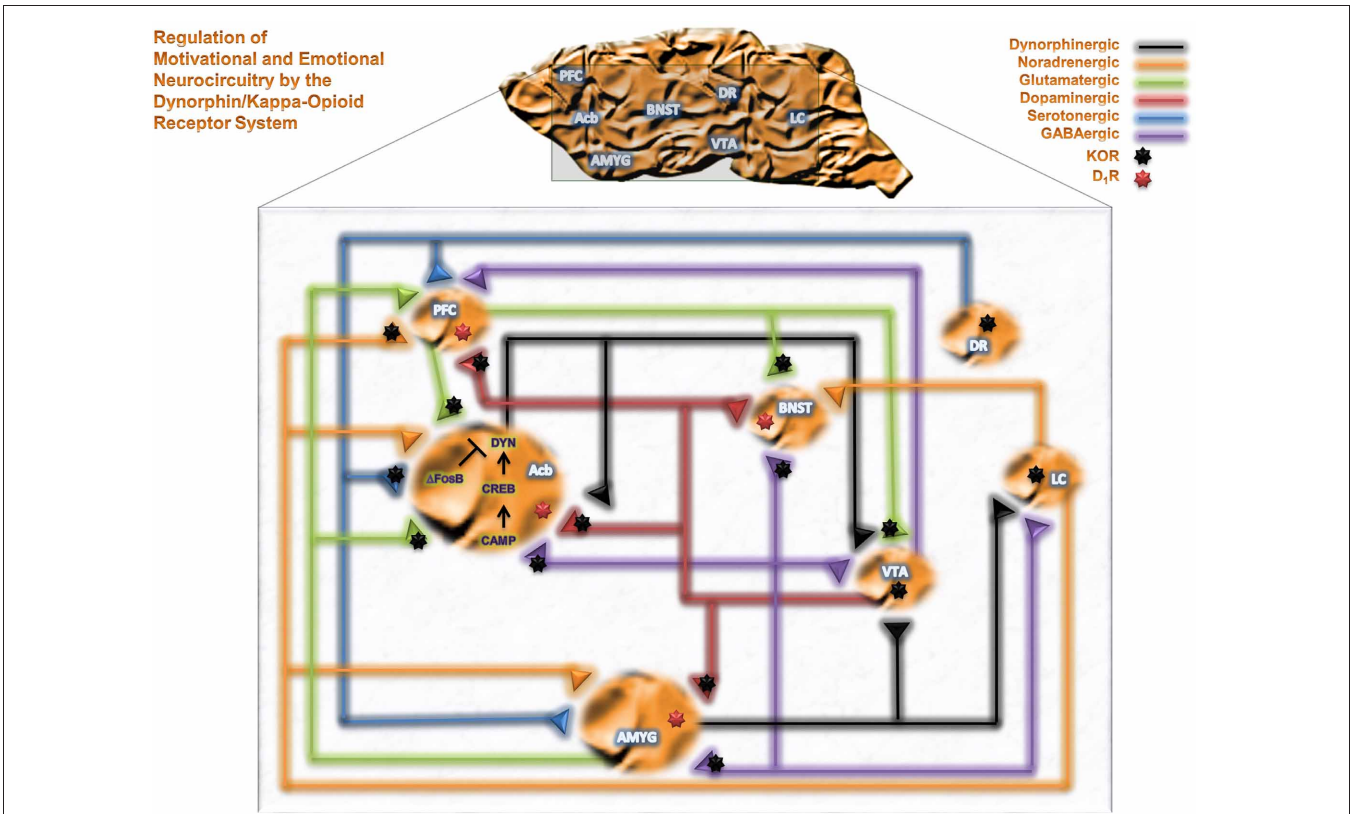

FIGURE 1 | The DYN/KOR system in motivational and emotional neurocircuitry. A schematic representation of brain motivational and emotional neurocircuitry, illustrating relevant neurotransmitters implicated in the pathophysiology of various neuropsychiatric disorders following chronic exposure of alcohol and drugs of abuse. Abbreviations: PFC, prefrontal cortex; Acb, nucleus accumbens; AMYG, amygdala; BNST, bed nucleus of the stria terminalis; VTA, ventral tegmental area; DR, dorsal raphe; LC, Locus coeruleus; GABA, γ-aminobutyric acid; DYN, dynorphin; cAMP, cyclic AMP, CREB, cAMP response element-binding protein; ΔFosB, a member of the Fos family of transcription factors.

within motivational and emotional circuitry (Heinz et al., 2009; Schwarzer, 2009).

ALCOHOL-INDUCED PLASTICITY IN THE DYN/KOR SYSTEM

Understanding the acute neurobiological effects of alcohol is critically important because once known, it may be possible to predict the neuroadaptive, and resulting, behavioral impact of long-term alcohol exposure using theories such as the opponent-process theory of motivation (OPT; Solomon and Corbit, 1974). If applying this theory to alcoholism, in order to maintain homeostasis, an increase in hedonic state (e.g., alcohol-induced euphoria) will be followed by a compensatory decrease in hedonic state (**Figure 2**). Furthermore, after repeated alcohol exposure, the positive hedonic state is reduced while the negative component is enhanced to compensate for the continued perturbation of the affective system by chronic alcohol exposure (**Figure 2**). As the cycle continues, the cessation of alcohol intake would result in the production of a negative affective state during withdrawal that can drive an organism to excessively seek and use alcohol. In accordance with the OPT, if alcohol-mediated MOR or DOR stimulation (Marinelli et al., 2004, 2005; Lam et al., 2008; Jarjour et al., 2009) produces positive hedonic states (Amalric et al., 1987; Shippenberg et al., 1987), then a compensatory mechanism could be increased DYN and/or function of KORs, stimulation of which produces negative hedonic states (Mucha and Herz, 1985). Under conditions of chronic alcohol use (see **Figure 2**), the predicted response of the endogenous opioidergic system would be attenuated MOR signaling and exacerbated DYN/KOR system activity, both of which are supported in the literature (Gianoulakis et al., 1996; Przewlocka et al., 1997; Turchan et al., 1999; Chen and Lawrence, 2000; Lindholm et al., 2000; Saland et al., 2004; Lindholm et al., 2007). In addition, chronic alcohol exposure has been shown to alter various neuropeptide systems (e.g., corticotropin-releasing factor (CRF), neuropeptide Y and nociceptin; Cowen and Lawrence, 2006; Ciccocioppo et al., 2009; Koob, 2010) that may contribute to the development of alcohol dependence and/or negative affective states. Therefore, a "counter-regulatory" the DYN/KOR system is recruited following exposure to alcohol and other drugs of abuse. Repeated alcohol exposure upregulates the DYN/KOR system and creates a state that facilitates alcohol seeking and consumption. The precise mechanisms that underlie escalated alcohol consumption in alcohol dependent states may involve adaptations at the pharmacological, transcriptional, and epigenetic levels and are discussed below.

PHARMACOLOGICAL EVIDENCE

It has been proposed that the neuroadaptive changes that occur in response to chronic alcohol use can occur via within- or between-system changes in reward and anti-reward systems, respectively (Koob and Bloom, 1988; Koob and Le, 2008; Koob, 2009). There is evidence supporting both possibilities in the form of neuroadaptations that occur within classical motivational systems (Koob and Weiss, 1992; McBride and Li, 1998; Siggins et al., 2003; Koob, 2004; Funk and Dohrman, 2007), as well as systems distinct from those that are involved in anhedonia and dysphoria (Valdez et al., 2002; Funk et al., 2006; Walker and Koob, 2008; Sperling et al., 2010; Nealey et al., 2011; Walker et al., 2011). Although DA and the EOS within the mesolimbic pathway, ventral striatum and CeA participate in dependence-induced within-system changes and stress-related peptides in the extended amygdala are hypothesized to participate in between-system changes [see the excellent review, (Koob, 2009)], there are several other neuropeptides involved in the positive and negative reinforcing effects of alcohol (Cowen and Lawrence, 2006; Koob and Le, 2008; Ciccocioppo et al., 2009; Gilpin and Roberto, 2012) that show great promise as therapeutics for the treatment of certain aspects of addictive disorders. Strong support for the OPT is recent evidence demonstrating that acute alcohol administration initially increases βEND within the first 30 min that is followed by a significant increase in DYN A approximately 1.5–2 h later (Lam et al., 2008; Jarjour et al., 2009); a profile that is also observed within the Acb and VTA (Marinelli et al., 2004, 2006; Jarjour et al., 2009). The OPT predicts that chronic alcohol exposure would decrease positive affect and increase negative affect (see **Figure 2**). In support of that prediction, evidence has shown that the MOR– and DOR–regulated component of the opioid system shows decreased signaling in response to chronic alcohol (Turchan et al., 1999; Chen and Lawrence, 2000; Saland et al., 2004). Also consistent with that hypothesis, chronic

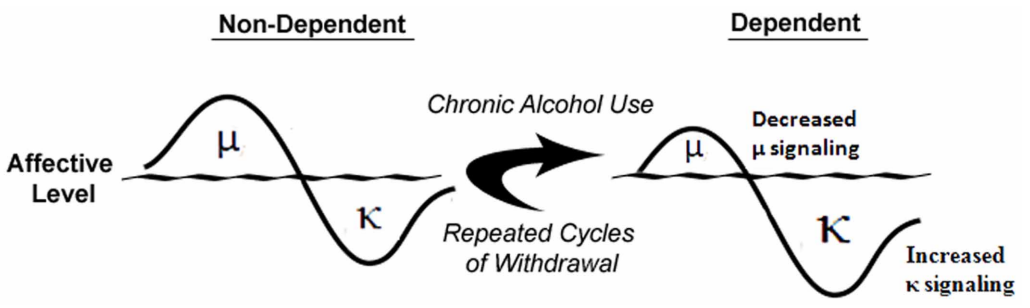

FIGURE 2 | Alcohol dependence-induced change in opioidergic system. In non-dependent organisms, alcohol-induced positive affective states mediated by the μ-opioid receptor precede compensatory negative affective states expressed through the κ-opioid receptor. Following chronic alcohol exposure, μ-opioid receptor signaling is attenuated and through multiple mechanisms, κ-opioid receptor signaling is increased to produce increased negative affective states. Adapted from Walker et al. (2012).

alcohol–exposed animals have been shown to have increased prodynorphin mRNA levels in the Acb (Przewlocka et al., 1997), increased expression of DYN B in the Acb (Lindholm et al., 2000) and altered KOR mRNA expression in the Acb and VTA (Rosin et al., 1999) that support the concept of an upregulated DYN/KOR system in these areas.

The functional impact of increased DYN/KOR system activity in dependence involves, in part, the mesolimbocortical DA system. This system has been implicated as a signaling system for biologically relevant information through which drugs of abuse (Koob, 2000; Maldonado, 2003; Di et al., 2004) and natural reinforcers (Hull et al., 1999; Carelli, 2002; Kelley et al., 2002, 2005) or punishers can exert their behavioral effects due to the mesolimbic DA pathway's capacity for bidirectional signaling (i.e., ability to signal both positive and negative stimuli; Wheeler and Carelli, 2009). Within the mesolimbic DA system, KORs are neuroanatomically positioned on DA terminals in the AcbSh that enables them to oppose the effects of MOR agonists on DA release (Di and Imperato, 1988). KORs are also positioned on DA perikarya in the VTA (Margolis et al., 2003, 2006). Much research has been done to determine how KOR stimulation impacts dopaminergic neurotransmission and drug self-administration, (for an excellent review, see Shippenberg et al., 2007). In essence, while the KORs positioned on the terminal regions in the AcbSh reduce DA release (Di and Imperato, 1988) in that region, KORs on VTA DA neurons do not (Spanagel et al., 1992; Margolis et al., 2006), but instead selectively reduce DA release in the PFC (Margolis et al., 2006). Thus, increased signaling through DYN/KOR system could functionally result in an attenuated dopaminergic system in both cortical and limbic circuitry. Indeed, deficiencies in dopaminergic transmission have been posited by some to be the neurobiological basis of depression (Nestler and Carlezon, Jr., 2006).

Substantial evidence supports the concept of chronic alcohol–induced attenuation of DA (Carroll et al., 2006; Healey et al., 2008). Stimulation of KORs produces dysphoria in humans (Pfeiffer et al., 1986) and place aversions in animals (Mucha and Herz, 1985). Furthermore, increased DYN transmission in the Acb has been hypothesized to induce depressive–like behavioral states in animal models of depression and negative affect (Pliakas et al., 2001; Mague et al., 2003; Carlezon, Jr. et al., 2006). The DYN/KOR system in the basolateral amygdala (BLA) has been implicated as a mediator of dysphoria through which stress–related systems can exert their effects (Land et al., 2008). Thus, if a compensatory response to chronic alcohol involved alterations in DYN/KOR signaling, then DYN/KOR–mediated negative affect could contribute to the increased alcohol consumption observed in dependence. Taken together, increased DYN transmission could result in attenuated dopaminergic transmission and produce depressive–like behaviors and dysphoria that are thought to involve multiple nuclei within extended Amyg circuitry. Therefore, if the DYN/KOR system is upregulated following chronic alcohol exposure in a manner sufficient to produce escalated alcohol self-administration (Roberts et al., 2000; O'Dell et al., 2004; Walker and Koob, 2008), KOR antagonists should be able to reduce negative affective states associated with withdrawal and reduce the excessive alcohol self-administration. Recent studies substantiating this hypothesis demonstrated that systemic, intracerebroventricular and intra-AcbSh administration of a KOR antagonist were able to selectively reduce escalated operant self-administration of alcohol in dependent Wistar rats while leaving nondependent alcohol self-administration intact (Walker and Koob, 2008; Nealey et al., 2011; Walker et al., 2011). These selective effects of KOR antagonists in dependent animals strongly implicate the recruitment of DYN/KOR system during the transition to alcohol dependence.

Although considerable work has focused on KOR modulation of DA transmission, KORs within the extended amygdala can presynaptically modulate other neurotransmitters (e.g., glutamate, GABA and serotonin; Fields et al., 2007; Land et al., 2009; Li et al., 2012). As many of these neurotransmitter systems have been implicated in alcohol reinforcement and dependence, upregulated DYN/KOR activity could be a common mechanism that adversely impacts motivational and emotional neurocircuitry. Specifically, KORs can presynaptically inhibit GABAergic signaling in the BNST that is thought to remove an important inhibitory influence on glutamatergic neurons and could contribute to the hyperglutamatergic state observed during alcohol withdrawal (Spanagel, 2009; Li et al., 2012). Furthermore, serotonergic projections from the dorsal raphe to limbic brain regions (Hensler, 2006) that can regulate affect and drug seeking behavior (Land et al., 2009; Bruchas et al., 2011) are impacted by alcohol exposure (Chu and Keenan, 1987; Pistis et al., 1997) and may be dysregulated during alcohol dependence (Gorwood et al., 2000; Shibasaki et al., 2010). KORs can also impact 5-HT levels via modification of efflux/tone (Tao and Auerbach, 2005) and appear to mediate aversive behavior in mice (Land et al., 2009). A recent study suggested that KOR mediated p38 MAPK signaling can induce serotonin transporter (SERT) translocation from the intracellular pool to the neuronal membrane, thereby enhancing serotonin reuptake (Land et al., 2009; Bruchas et al., 2011) and causing a hyposerotonergic state. Furthermore, Shibasaki and colleagues have shown upregulated SERT mRNA in the dorsal raphe of alcohol-dependent rodents (Shibasaki et al., 2010). Taken together, DYN/KORs in this circuitry may be upregulated in alcohol dependence and may reduce serotonergic tone through a p38 MAPK-dependent SERT translocation mechanism that can contribute to the development of a negative affective state. Modulation of this state has been shown to manage certain symptoms of rapid alcohol exposure (Johnson, 2004; Uzbay, 2008), although the face and predictive validity of the models used to evaluate alcohol withdrawal should be carefully scrutinized. Another mechanism through which KORs can regulate affect was identified by Hjelmstad and Fields (2001) who documented that KORs are also anatomically positioned on presynaptic terminals of glutamatergic inputs from the PFC, Amyg and hippocampus to the Acb. Therefore, in addition to local blockade of DA release in the Acb by DYN/KOR, KOR modulation of glutamatergic inputs to the Acb may also be involved with aversive behaviors; however, additional studies are needed to confirm this. These studies may help to explain the significant comorbidity between alcohol use disorders and affective disorders (Regier et al., 1990; Grant and Harford, 1995) that can plague those afflicted with alcohol dependence.

The EOS may also have a role in certain cognitive processes relevant for control of addictive behavior including craving, decision-making and impulsivity (O'Malley et al., 2002; Bencherif et al., 2004; Boettiger et al., 2009; Love et al., 2009). Thus, a dysregulated EOS may contribute to impaired neurocognitive function and reduced regulation of alcohol/drug seeking and consumption. A recent study demonstrated a significant increase in PDYN mRNA (PDYN is the precursor peptide for the two forms of DYN, DYN A, and DYN B) in the dorsolateral prefrontal cortex (dl-PFC), and KOR mRNA in the orbitofrontal cortex (OFC) of deceased alcoholics when compared to controls (Bazov et al., 2011; Taqi et al., 2011b). Furthermore, levels of both DYN A and DYN B were significantly elevated in the dl-PFC and hippocampus of alcoholics. Importantly, the levels of PDYN mRNA significantly correlated with those of DYN peptides. These alterations were observed in brain regions involved in cognitive control of addictive behavior. Therefore, DYNs may have a role in regulation of executive functions and their elevation may impair these cognitive processes. In addition, DYN A can induce effects that are not blocked by opioid antagonists (Faden and Jacobs, 1983; Dubner and Ruda, 1992; Caudle and Mannes, 2000; Lai et al., 2001; Tan-No et al., 2001, 2005; Singh et al., 2003; Hauser et al., 2005). These non-canonical non-opioid effects, generally excitatory, may lead to neurodegeneration and pathological behavior such as chronic pain and paralysis. Therefore, both the opioid-receptor mediated and non-opioid neurodegenerative mechanisms may underlie the behavioral effects of upregulated DYN/KOR system in alcoholics.

TRANSCRIPTIONAL EVIDENCE

Following DA release in motivational nuclei (i.e., Acb), it binds to its receptors (D_1- or D_2-like) and produces excitatory or inhibitory post-synaptic potentials, respectively. D_1-like receptor activation increases adenylyl cyclase activity by coupling to stimulatory G proteins (Gαs). This leads to an increase in the concentration of cyclic AMP (cAMP) and potentiation of cAMP-dependent protein kinases A (PKA) activity that further phosphorylates downstream signaling substrates. One signaling substrate is the transcription factor cAMP response element-binding protein (CREB) that activates the transcription of *PDYN*, *BDNF*, *CRF*, *NPY,* and other genes (Lonze and Ginty, 2002; Carlezon, Jr. et al., 2005). CREB-mediated increases in DYN within the Acb serves as a negative feedback circuit whereby it decreases DA release in Acb through presynaptic receptors on DA containing nerve terminals (see **Figure 1**). This is evident from experiments showing that KOR antagonist blocks the effects of CREB overexpression (Carlezon, Jr. et al., 1998). Because alcohol exposure may alter CREB mediated pathway in the Acb (Misra et al., 2001), mice lacking a regulatory subunit of PKA show attenuated cAMP-PKA signaling in the Acb and increased alcohol consumption (Thiele et al., 2000). In addition, PKA inhibition, that further decreases PKA/CREB activity, produces high alcohol preference in rodents (Misra and Pandey, 2006). Activation of CREB results in elevation of DYN levels in this circuitry (Carlezon, Jr. et al., 1998). Upregulated CREB signaling in the VTA-Acb pathway produces pro-depressive behavior (Pliakas et al., 2001; Malberg and Blendy, 2005), while CREB or DYN inhibition in the Acb produces an antidepressant-like effect (Newton et al., 2002). Early exposure to methylphenidate that causes sustained elevation in CREB in Acb produces anhedonia and dysphoria (Bolanos et al., 2003; Carlezon, Jr. et al., 2003). Collectively, CREB-mediated DYN upregulation in the Acb may mediate reduced reward and pro-depressive effects of chronic alcohol and drug exposure. In the Amyg, alterations in CREB-mediated signaling following alcohol exposure has been implicated in anxiety associated with alcohol withdrawal, albeit in a direction opposite of that in the Acb (Pandey et al., 2005); however, the time-points of CREB evaluation in the Amyg (although during acute withdrawal) were more protracted than in other investigations (24 h vs. 6–10 h into withdrawal; (Pandey et al., 2005; Williams et al., 2012, respectively). Thus, CREB-mediated increases in DYN within critical brain regions implicated in mood regulation may contribute to the development of negative affective behavior during withdrawal in dependent organisms.

The cAMP signaling pathway is one mechanism by which alcohol and other drugs of abuse can alter DYN concentrations in the Acb via CREB-mediated transcriptional activation. In addition to directly targeting the prodynorphin gene, CREB may regulate several signaling pathways (Carlezon, Jr. et al., 2005) that may activate the DYN/KOR system following chronic exposure to alcohol or addictive drugs (Carlezon, Jr. et al., 2005; Nestler and Carlezon, Jr., 2006). Brain- derived neurotropic factor (BDNF) is an important CREB target (Lonze and Ginty, 2002) that modulates DYN expression in brain region specific manner (Nair and Vaidya, 2006). BDNF, a member of the nerve growth factor (NGF) family, and its receptor TrkB are widely distributed throughout the brain (Wetmore et al., 1990; Altar et al., 1994), and have a role in synaptic plasticity (Chao, 2003) associated with alcohol addiction (Uhl et al., 2001) and several psychiatric disorders (Martinowich et al., 2007). BDNF expression is associated with early onset of alcoholism (Matsushita et al., 2004). Alcohol exposure increases BDNF expression in the dorsal striatum (DS) leading to prodynorphin activation (Logrip et al., 2008). These effects are brain region specific (Nair and Vaidya, 2006) and further work is needed to understand the precise pathophysiological mechanisms of dysregulated CREB-BDNF signaling in alcohol dependence. Thus, alcohol and other substances of abuse may dysregulate CREB-mediated signaling in the Acb leading to DYN upregulation. That upregulation appears to contribute to the depressive and aversive effects of alcohol, cocaine and other illicit drugs (Carlezon, Jr. et al., 1998, 2005; McClung and Nestler, 2008).

One transcription factor that gained attention for its putative role in long-lasting plastic changes underlying addiction is ΔFosB (Nestler et al., 2001). ΔFosB is a member of the Fos family of transcription factors encoded by the *FOS* genes. Fos proteins are rapidly, but for a short period of time, induced in Acb and dorsal striatum, brain regions involved in the rewarding and locomotor effects of various drugs of abuse and alcohol (Nye and Nestler, 1996; Kelz et al., 1999; Kelz and Nestler, 2000; Perrotti et al., 2008). Following chronic treatment with alcohol or drugs of abuse only ΔFosB has been found to accumulate in many brain regions related to goal-directed actions and decision-making, including the striatum and PFC (Hope et al., 1994; Kelz and Nestler, 2000; Perrotti et al., 2008). ΔFosB is an unusually stable, C-terminally truncated variant of the immediate early gene product FosB. ΔFosB is thought to function as a

sustained molecular switch for addiction. Chronic exposure of rodents to most drugs of abuse, including cocaine, morphine, Δ9-tetrahydrocannabinol, and alcohol, causes ΔFosB to accumulate in addiction-related circuitry (Perrotti et al., 2008), wherein it has been suggested to regulate the expression of several genes commonly associated with this disease (Nestler, 2008; Robison and Nestler, 2011). ΔFosB regulates gene expression via formation of the AP-1 complex that is critically involved in regulation of neuronal activity and behavior after long period of withdrawal (Nestler et al., 2001). ΔFosB may be selectively induced in DYN/substance P containing neurons (Moratalla et al., 1996). Expression of several genes, including *PDYN*, may be regulated by ΔFosB. It has been hypothesized that ΔFosB-mediated suppression of DYN expression in Acb may increase the sensitivity for the rewarding effects of drugs of abuse (Zachariou et al., 2006). These effects may oppose CREB-mediated signaling and ΔFosB is thought to be involved in maintaining addiction-related changes in neurophysiology by: (1) enhancing the rewarding and incentive motivational properties of drugs of abuse via its actions in the Acb and (2) producing tolerance to the cognitive-disrupting effects of such drugs via its actions in the PFC. Relatively short-lived CREB-DYN activity and persistent ΔFosB effects might explain some CREB-DYN mediated behaviors (e.g., tolerance, negative affective states, and depression-like behavior) during early stages of alcohol withdrawal. Alternatively, ΔFosB/DYN-mediated effects may be important during later stages of abstinence from alcohol or drug of abuse (Nestler et al., 2001).

Transcriptional models of addiction involving CREB and ΔFosB gained support in pharmacological and genetic experiments with rodents. However, the promoter and enhancer structure of many human and rodent genes that are implicated in addictive behavior (including *PDYN)* are not completely conserved across species. To address the ΔFosB hypothesis in the context of substance dependence in humans, FosB proteins in human brain were characterized by analysis of postmortem specimens, and compared their levels in the OFC and dlPFC, respectively between human controls and alcoholics (Watanabe et al., 2009). These two sub-regions of the larger PFC are important nuclei within addiction neurocircuitry and chronic exposure to alcohol or other drugs of abuse has been shown to result in accumulation of ΔFosB in both structures (Winstanley et al., 2007; Perrotti et al., 2008). In both the dl-PFC and OFC, as well as in the Acb, three forms of FosB were detected, one of which was ΔFosB. The later protein was found to be expressed at very low, barely detectable levels in all three human brain regions. Importantly no differences in ΔFosB levels were evident between alcoholics and control groups. These human results do not support the ΔFosB hypothesis; they indicate that ΔFosB does not accumulate in the OFC and dlPFC of human alcoholics, suggesting that it may not be directly involved in addiction maintenance, at least not in alcohol dependence.

AP-1 may potentially regulate transcription of human prodynorphin gene in the human brain. Analysis of AP-1 constituents in the human brain demonstrated that canonical and noncanonical prodynorphin AP-1-binding element may be targeted by c-Jun and FosB proteins that form the dominant AP-1 complex (Taqi et al., 2011a). No ΔFosB was found in such a complex. Nonetheless, transcription of human *PDYN* may be regulated by AP-1 forming JunD/FosB heterodimers that binds to a noncanonical AP-1-binding element. This element has a polymorphic site with the T allele conferring AP-1 binding. The C allele of this single nucleotide polymorphism (SNP; rs1997794) that destroys this site represents the allele that is associated with alcohol dependence. Thus, a SNP in the promoter of *PDYN* is associated with alcohol-dependence and may impact *PDYN* transcription in human brain. The impact of genetic variations on *PDYN* transcription may be relevant for diverse adaptive responses of this gene to alcohol.

EPIGENETIC EVIDENCE

Pleasurable and adverse states resulting from the intake of alcohol and addictive drugs can shape individual differences in the vulnerability to addictive disorders. A fundamental question is how these experiences are encoded at a molecular level in a manner that leads to long-term alterations in plasticity that underlie increased risk for substance abuse. Dysregulation of epigenetic mechanisms could lead to silencing or inappropriate expression of specific genes that could contribute to the pathologies observed in those who are alcohol dependent.

Epigenetics is typically defined as the study of heritable changes in gene expression that are not due to changes in DNA sequence (Eccleston et al., 2007). Hence, identical DNA sequences with differential epigenetic regulation could result in differential gene expression. Epigenetic regulation seems to be time and tissue specific and can be quite diverse even within the same tissue or individual. Epigenetic changes represent alterations in gene expression that are self-perpetuating in the absence of the original signal that caused them (Berger et al., 2009). Environmental factors, including alcohol, can modulate gene expression by inducing alterations in epigenetic markers such as DNA methylation and histone modifications. Epigenetic changes have been associated with a range of neurobiological processes including brain development, synaptic plasticity, learning and memory and neuropathologies such as drug addiction (Borrelli et al., 2008; Renthal and Nestler, 2008; Roth and Sweatt, 2009). DNA methylation is the most stable epigenetic mark that is responsive to environmental stimuli. Environmental conditions can evoke changes in DNA methylation underlying epigenetic re-programming of genes involved in the regulation of addictive disorders. Effects of genetic variations including single nucleotide polymorphisms (SNPs) on DNA methylation may depend on DNA context (Kerkel et al., 2008; Xie et al., 2009; Hellman and Chess, 2010; Shoemaker et al., 2010; Zhang et al., 2010). Importantly, polymorphic positions are most abundant at the CpG dinucleotides that are targets for DNA methylation (Tomso and Bell, 2003; Kerkel et al., 2008; Xie et al., 2009; Hellman and Chess, 2010; Shoemaker et al., 2010; Zhang et al., 2010).

The Bakalkin group has recently addressed the specific hypothesis that genetic, epigenetic and environmental factors associated with a risk for addictive disorders mechanistically converge on SNPs that (1) are associated with addiction, and (2) that overlap with CpG sites thus representing methylation-associated SNPs, or mSNPs (**Figure 3**) (Taqi et al., 2011b). The two epialleles formed by the unmethylated and methylated C allele at such mSNPs may differentially contribute to disease predisposition, as they may be targeted by transcription factors or by insulator proteins that

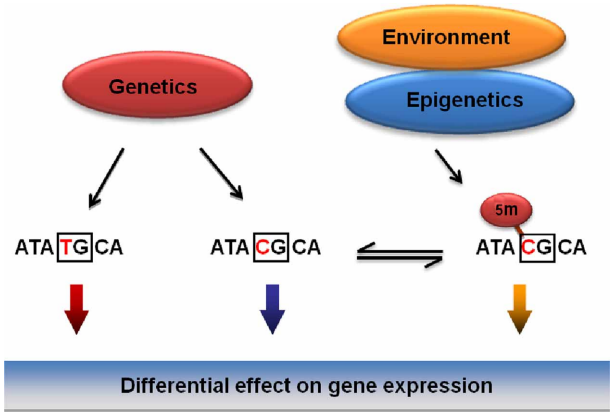

FIGURE 3 | General model for the integration of genetic, epigenetic and environmental factors on vulnerability to develop addictive disorders. Mechanistically, the effects of these factors may be integrated through methylation of CpGs overlapping with SNPs (mSNPs) associated with a disease. In the CpG context, the C, one of the two genetic alleles may be methylated and function as two epialleles, the C and ^{5}Me-C, that may differentially affect gene expression.

control the interactions among genomic regulatory elements (for insulators, see (Wallace and Felsenfeld, 2007). This hypothesis (1) unifies the genetic and epigenetic views on the vulnerability to develop addictive disorders, and (2) may explain a part of "missing heritability" not detected in the genome-wide association studies (GWAS).

Our analysis of *PDYN* regulation in the brains of deceased human alcoholics (Taqi et al., 2011b) demonstrated that three *PDYN* SNPs significantly associated with alcohol dependence form CpG sites, and that methylation of one of them was increased and positively correlated with DYN in alcoholics. The mSNP hypothesis has also received support in several recent studies (John et al., 2011; Kaminsky et al., 2012; Martin-Trujillo et al., 2011; Reynard et al., 2011; Ursini et al., 2011). Thus, under influences of the environment—heavy alcohol drinking-induced alterations in methylation of this SNP may affect *PDYN* transcription and, consequently, the vulnerability to develop alcohol dependence. These alterations were observed in brain regions involved in cognitive control of decision-making and may represent molecular adaptations that developed after many years of alcohol exposure and withdrawal. DYN may have a role in the regulation of executive and intellectual functions, learning and memory, and emotions in alcoholics; therefore, their elevation may impair these cognitive processes. Taken together, the results suggest that epigenetic plasticity in the DYN/KOR system may be involved in mediating some of the behavioral effects produced following chronic alcohol exposure.

CONCLUSION

The goal of this review is to discuss alcohol-induced plasticity in the DYN/KOR system and how these neuroadaptations may contribute to the pathophysiology of alcohol dependence. The DYN/KOR system has been implicated as an endogenous anti-reward system. However, an upregulated DYN/KOR system in various key brain regions at proximal (DYN/KOR mRNA and expression) and intermediate (CREB/ΔFosB/BDNF mediated signaling) levels could contribute to altered distal events (escalated alcohol use, affective/anxiety like behaviors, sensitization following abstinence) in alcohol dependence. Therefore, increased DYN/KOR activity may induce a negative affective state in withdrawal and provide a basis for the negative reinforcing effects of alcohol. DYN/KORs in the Acb, Amyg and PFC/OFC may mediate the effects of chronic alcohol exposure in a brain region specific manner to decrease positive hedonic states, increase negative affective states or impair decision making and cognitive control, respectively. In addition, epigenetic mechanisms may also be involved in the upregulation of the DYN/KOR system following chronic alcohol exposure. Recent data supporting the role of the DYN/KOR system in mediating negative affective states also help to explain significant co-morbidity between alcohol use disorders and affective disorders. Taken together, the DYN/KOR system is heavily dysregulated in alcohol dependence and represents a potential therapeutic target to combat alcoholism.

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Neuronal nicotinic acetylcholine receptors: neuroplastic changes underlying alcohol and nicotine addictions

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Addictive drugs can activate systems involved in normal reward-related learning, creating long-lasting memories of the drug's reinforcing effects and the environmental cues surrounding the experience. These memories significantly contribute to the maintenance of compulsive drug use as well as cue-induced relapse which can occur even after long periods of abstinence. Synaptic plasticity is thought to be a prominent molecular mechanism underlying drug-induced learning and memories. Ethanol and nicotine are both widely abused drugs that share a common molecular target in the brain, the neuronal nicotinic acetylcholine receptors (nAChRs). The nAChRs are ligand-gated ion channels that are vastly distributed throughout the brain and play a key role in synaptic neurotransmission. In this review, we will delineate the role of nAChRs in the development of ethanol and nicotine addiction. We will characterize both ethanol and nicotine's effects on nAChR-mediated synaptic transmission and plasticity in several key brain areas that are important for addiction. Finally, we will discuss some of the behavioral outcomes of drug-induced synaptic plasticity in animal models. An understanding of the molecular and cellular changes that occur following administration of ethanol and nicotine will lead to better therapeutic strategies.

Keywords: addiction, behavioral sensitization, cholinergic, ethanol, neuroplasticity, nicotine, nicotinic acetylcholine receptors, synaptic transmission

INTRODUCTION

For the past several decades researchers have put forth concerted efforts to investigate the effects of drugs of abuse on brain function with the ultimate goal of developing medications useful in terminating drug use and preventing relapse. The progression from initial drug use to drug dependence involves complex, multifaceted neural adaptations that encompass molecular changes at the cellular level within several different brain circuits. From studies involving human subjects and animal models, drugs of abuse are known to act on the cortico-limbic network (see **Figure 1**) that governs reward (mesolimbic dopaminergic pathway), learning and memory (hippocampus), emotion (amygdala), and executive functions (prefrontal cortex). Our understanding, at this point, remains limited regarding how drug-related experiences are encoded in the brain and their relationship to synaptic plasticity and the development of addictions.

It is now generally accepted that addiction is a type of learning, i.e., learned associations between the rewarding effects of drugs and environmental cues that predict drug availability, which significantly contributes to compulsive drug use and the propensity to relapse even after long periods of abstinence. As subjects make the transition from initiation to habitual drug use, neuroadaptations in areas such as the hippocampus and amygdala are thought to underlie drug-associated learning and memories. A single episode of drug use itself can influence synaptic transmission and repeated or prolonged drug use can cause long-lasting alterations in synaptic strengths—defined as synaptic plasticity, reflected through molecular changes as well as persistent modification of neurotransmitter release. These drug-induced changes in the brain are a critical component in the development of dependence and are thought to drive compulsive intake and relapse. There are many factors that play into addictive processes and understanding the neurobiological underpinnings of these events will enlighten possible therapeutic targets.

The most well-known form of synaptic plasticity, NMDAR-dependent, was first discovered in 1973 in the hippocampi of anesthetized rats (Bliss and Lomo, 1973) and has since been extensively characterized from electrophysiological recordings of *in vitro* hippocampal slice preparations. Long-term potentiation (LTP) is defined as an increase in the post-synaptic response resulting from a cascade of events which is initiated by an influx of Ca^{2+} ions through voltage-gated pre-synaptic ion channels. The NMDAR-dependent form of LTP requires postsynaptic depolarization during NMDAR activation, which will allow for an influx of Ca^{2+} ions through the channel within the dendritic spine. This increase of Ca^{2+} will commence a series of intracellular signaling, activating a number of protein kinases and consequently lead to the insertion of AMPA receptors into the plasma membrane. In contrast, long term depression (LTD) is attributed to a weak activation of NMDARs, minimal Ca^{2+} influx, and a reduction in post-synaptic AMPA surface receptor density via dynamin- and clathrin-dependent endocytosis (Malenka and Bear, 2004).

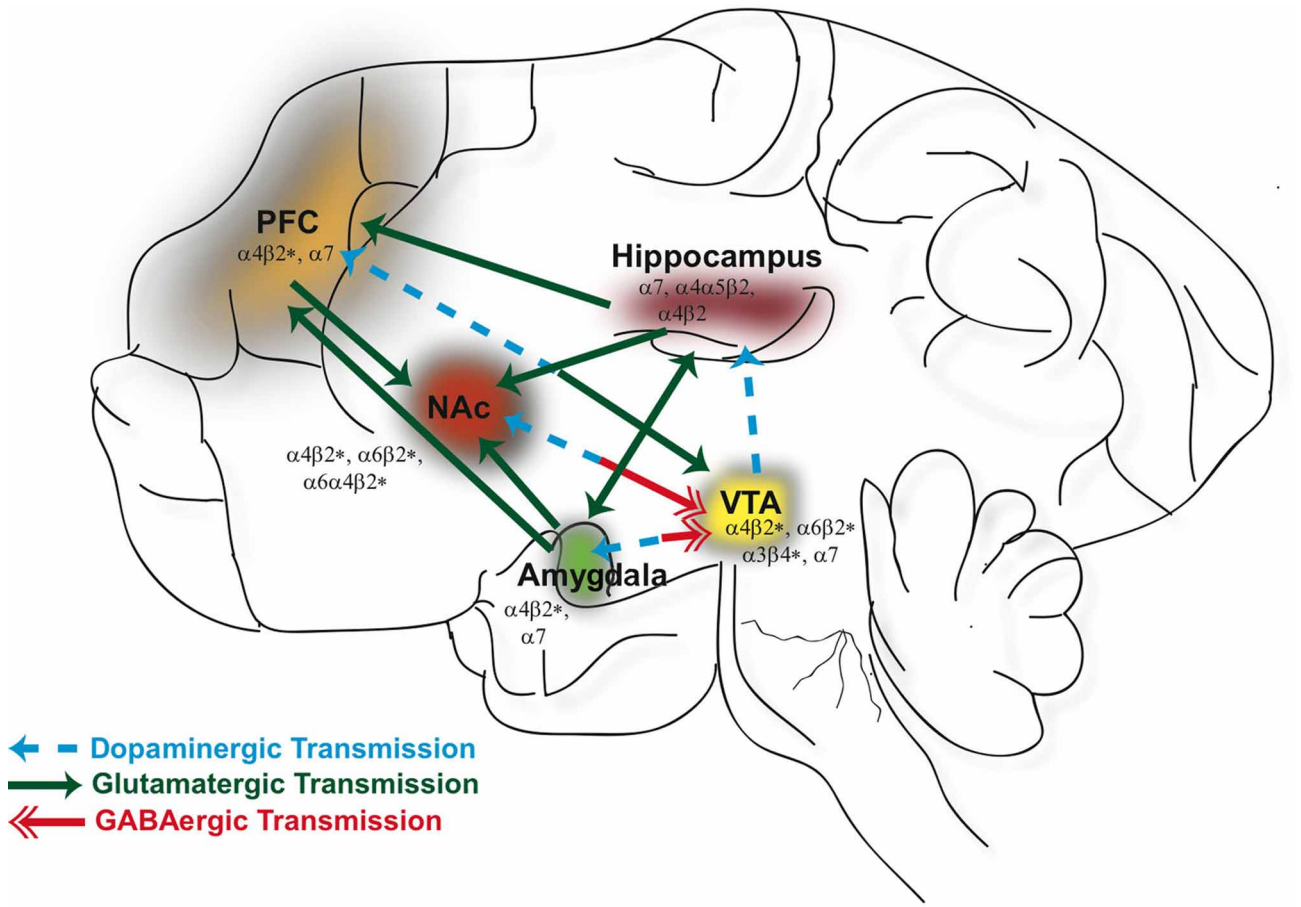

FIGURE 1 | Neuronal nicotinic acetylcholine receptors (nAChRs) are widely distributed in different brain regions that include the ventral tegmental area (VTA), nucleus accumbens (NAc), hippocampus, prefrontal cortex (PFC), and amygdala. Activation of nAChRs in these brain areas significantly contribute to the rewarding effects of ethanol and nicotine and play a role in modulating synaptic plasticity. GABAergic (red), glutamatergic (green), and dopaminergic (blue) connections between these structures constitute a major neural circuitry underlying addictive disorders.

Much of the NMDAR-dependent plasticity has focused on mechanisms responsible for the initial increase in synaptic strength, however the long-lasting biological effects likely require new protein synthesis and gene transcription (Lynch, 2004). There are quantifiable alterations in the morphology of dendrites and dendritic spines that accompany LTP (Andersen and Soleng, 1998; Yuste and Bonhoeffer, 2001; Matsuzaki et al., 2004) and together these long-lasting changes have been implicated as an essential mechanism and molecular basis for learning and memory. Additionally, there are other modulators of plasticity, such as metabotropic glutamate receptors and endocannabinoids, that have been discovered and reviewed extensively elsewhere [see review, Kauer and Malenka (2007)].

Alcohol and tobacco addiction are among the highest causes of preventable death worldwide (Mokdad et al., 2004) and the comorbidity of these two substance abuse disorders is striking (DiFranza and Guerrera, 1990; Batel et al., 1995; Falk et al., 2006). While the easy availability and low social stigma of alcohol and cigarettes provides an explanation of their high prevalence of dual dependence, strong neurobiological evidence suggests a common link between these two substances (de Fiebre et al., 1990; Smith et al., 1999; Gould et al., 2001; Marubio et al., 2003; Tizabi et al., 2007). Neuronal nicotinic acetylcholine receptors (nAChRs) are widely expressed throughout the brain (Gotti et al., 2007) and are suggested to be the common biological target of nicotine and ethanol (Tapper et al., 2004; Funk et al., 2006; Steensland et al., 2007; Bito-Onon et al., 2011). nAChRs are pentameric ligand-gated ion channels, consisting of various heteromeric or homomeric combinations of α (α_2–α_{10}) and β (β_2–β_4) subunits (Albuquerque et al., 2009; Gotti et al., 2009). Most neuronal nAChRs are heteromeric receptors with just two binding sites, but some subunits, such as the α7, form functional homomeric receptors with five binding sites (Changeux, 2009; Gotti et al., 2009). The most abundant of nAChR subtypes in the brain are the α4β2* (*indicates the possibility of other subunits) followed by the α7; correspondingly, the mRNA of these subtypes are found throughout the entire brain. The vast regional distribution and location of nAChRs are thoroughly reported in the following reviews (Gotti and Clementi, 2004; Gotti et al., 2007).

Binding of endogenous acetylcholine (ACh) or nicotine induces a conformational change of the receptor allowing for an influx of cations (Ca2+, Na+, or K+ depending on nAChR subtype) through the central channel for a few milliseconds, followed by a non-conducting closed receptor state (Giniatullin et al., 2005). In contrast, ethanol is not a direct agonist at nAChRs but can potentiate the response of these receptors to Ach (Aistrup et al., 1999; Cardoso et al., 1999; Zuo et al., 2002). The pharmacological properties of the nAChRs to agonists such as ACh, nicotine or ethanol is highly dependent on its subunit composition and location of the receptor (Yu et al., 1996; Pidoplichko et al., 1997; Cardoso et al., 1999; Wooltorton et al., 2003).

Nicotinic receptors are localized both pre- and post-synaptically where agonist-induced cation influx results in membrane depolarization and/or CA^{2+}-dependent signaling cascades, thereby regulating neuronal excitability and neurotransmitter release (Albuquerque et al., 1995). nAChRs play a significant role in modulating glutamatergic, GABAergic, and dopaminergic neurotransmission in the mesolimbic pathway (Blomqvist et al., 1992; Caille et al., 2009; Mao et al., 2011), thus contributing to the rewarding effects of drugs of abuse and synaptic plasticity in this system as well as in other brain regions such as the hippocampus and amygdala (Ericson et al., 2009; Hendrickson et al., 2010; Reperant et al., 2010). Moreover upon exposure to nicotine and ethanol, nicotinic receptors can undergo changes in expression (stoichiometries or receptor number) and function which may underlie aspects of physical dependence and withdrawal symptoms (Nashmi et al., 2007). Since the majority of alcoholics are also smokers, determining the coincident molecular underpinnings in the development of their dependence may be useful in treating these addictions.

This review will attempt to consolidate the available information regarding nicotinic receptor-mediated synaptic plasticity and integrate these enduring neural adaptations with current models of addictive disorders. We will look at a myriad of addictive processes, the underlying neural circuits and how these pathways converge for addictive behaviors to emerge. The complexity of addictive disorders suggests there are a considerable number of possible targets for intervention that could potentially reverse drug-induced neural adaptations. We will aim to focus this paper towards nicotinic receptor-mediated neuroplasticity, with an emphasis on nicotine and ethanol.

NICOTINE AND ETHANOL: nAChR-MEDIATED NEUROTRANSMISSION AND PLASTICITY

In addition to alterations in glutamatergic signaling, there is ample evidence showing other neurotransmitter systems, such as the cholinergic and dopaminergic, play a vital role in modulating the induction, duration, and magnitude of synaptic plasticity (Otani, 2003; Drever et al., 2011). ACh acts on a variety of different pre- and post-synaptic receptors throughout the brain resulting in profoundly different outcomes depending on receptor location and subunit composition (Alkondon and Albuquerque, 2004). Thus far, most studies have implicated the involvement of G-protein coupled muscarinic receptors in mediating these synaptic changes; however, more recently nAChRs have come under investigation to understand their part in these processes. In this section, the role of nAChRs in the modulation of neurotransmission and drug-induced plasticity will be discussed for the brain loci that have been implicated to be important for the development of nicotine and ethanol addiction.

MIDBRAIN: REWARD PATHWAY

The mesolimbic dopaminergic system, encompassing DA neurons originating from the ventral tegmental area (VTA) that project to the nucleus accumbens (NAc) and PFC, has long been recognized as an important pathway mediating behavioral responses to natural rewards as well as drugs of abuse. Essentially, all drugs of abuse enhance extracellular DA in the NAc (Di Chiara and Imperato, 1988) and blocking dopaminergic transmission will attenuate the reinforcing properties of the drug (Corrigall et al., 1992). Furthermore, this DA signal provides convergent information to the system regarding reward expectation and environmental cues related to drug intake (Di Chiara, 1999; Berke and Hyman, 2000). It is not surprising, therefore that drug-induced neural adaptations have been discovered in this circuit and presumably contribute to addiction.

Ventral tegmental area

The VTA is modulated by excitatory glutamatergic inputs arising from the PFC, bed nucleus of the stria terminalis, amygdala, pontomesencephalic tegmental nuclei (Mao and McGehee, 2010), and by a large population of inhibitory GABAergic interneurons (Johnson and North, 1992; Theile et al., 2008) and afferents that arise from heterogeneous sources (Geisler and Zahm, 2005). There are several different nAChR subunits expressed in the VTA, some of which are the α3, α4, α5, α6, α7, β2, and β3 subtypes (Azam et al., 2002; Perry et al., 2002; Yang et al., 2009). The α4 and β2 mRNAs are expressed in nearly all DAergic and GABAergic VTA neurons, while α7 mRNA is distributed in only 40% of these neurons (Nashmi and Lester, 2006). The α7* nAChRs are most densely localized on pre-synaptic glutamatergic, but not cholinergic, terminals in the VTA (see **Figure 2**) (Klink et al., 2001; Jones and Wonnacott, 2004).

Both ethanol and nicotine stimulate dopamine release in the accumbens by modulating the activity of VTA neurons via nAChRs (Champtiaux et al., 2003). In the presence of physiologically relevant doses of nicotine (100–500 nM) (Nguyen et al., 2003; Parker et al., 2004), nAChRs are briefly activated followed by rapid desensitization, which entails a reversible inactivation of response. Therefore, nicotine's mechanism of action and downstream consequences are attributed to both receptor activation and desensitization (Mansvelder et al., 2002). The propensity of these receptors to desensitize depends largely on the subunit composition of the nAChR assembly. α7 nAChRs have a much lower affinity for nicotine compared to β2-containing nAChRs and are less susceptible to desensitization in the presence of relevant smoking-related concentrations (Mansvelder and McGehee, 2002; Quick and Lester, 2002; Wooltorton et al., 2003).

The initial few minutes of nicotine exposure will enhance DA release in the NAc by activating α4β2* nAChRs on dopamine neuronal soma (Pidoplichko et al., 1997; Mansvelder and McGehee,

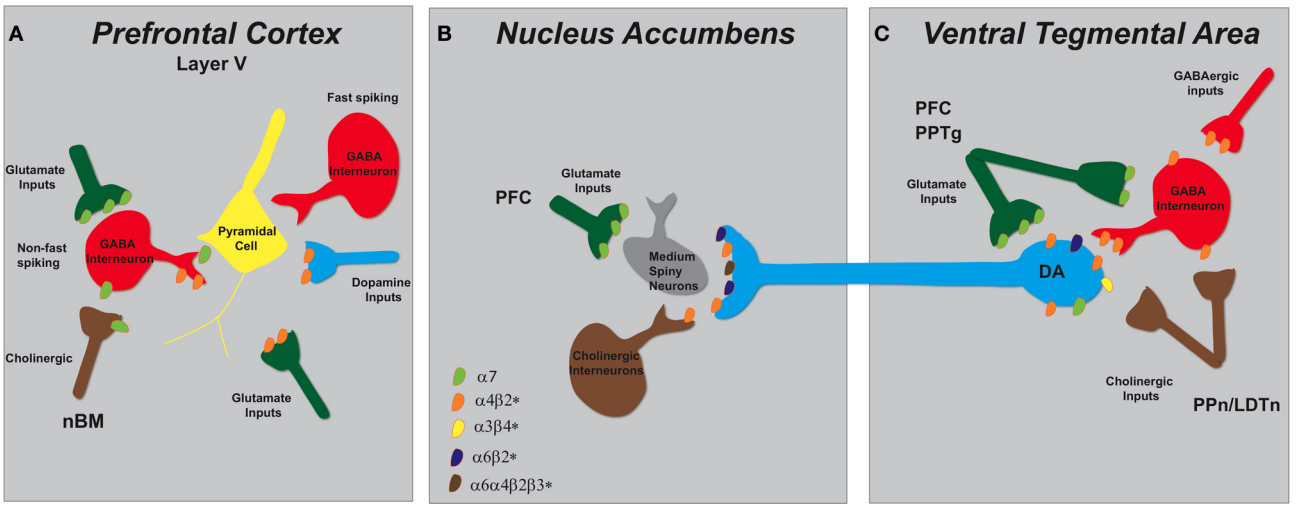

FIGURE 2 | Schematic representation of nAChR subtypes and circuit function in the mesolimbic dopaminergic system. (A) Pyramidal cells in layer V of the PFC lack nAChRs but their activity is modulated by excitatory and inhibitory neurons that do express them. There are two types of GABAergic interneurons, fast spiking and non-fast spiking, with only the latter bearing nAChRs (α7 and α4β2*). Distinct populations of glutamatergic inputs express either α7 or α4β2* nAChRs while DA terminals projecting from the VTA contain α4β2* nAChRs. Cholinergic inputs into the PFC arise from the nucleus basalis of Meynert (nBM). **(B)** In the NAc, nAChRs (α4β2*, α6β2*, and α6α4β2*) expressed on DAergic terminals from the VTA mediate DA release based on the neuronal activity firing rate. A small population of tonically active cholinergic interneurons (~2%) is synchronized with DA cell firing. Glutamatergic inputs from the PFC endow α7 nAChRs. **(C)** The VTA receives cholinergic innervation from the pedunculopontine (PPn) and laterodorsal tegmental nuclei (LDTn). In addition to the nAChRs localized on DA cell bodies, DAergic cell firing is modulated by α4β2* (and possibly α7) nAChRs expressed on GABAergic interneurons and excitatory glutamatergic afferents from the PFC and the PPn.

2000); however, β2*-containing receptors rapidly desensitize. Simultaneously, nicotine acts on α4β2* nAChRs located on GABAergic neurons to induce a transient inhibition of DA activity. These nicotinic receptors will also subsequently desensitize in the presence of nicotine with a net result being a reduction in the inhibitory control of GABA on dopaminergic transmission (Mansvelder and McGehee, 2002). On the same time scale, nicotine binds to α7 nAChRs present on glutamatergic terminals in the VTA, whose activation increases glutamate release onto NMDA-type glutamate receptors located on dopaminergic cell bodies and increases the frequency of spontaneous excitatory postsynaptic currents (sEPSCs) (Mansvelder and McGehee, 2000; Schilstrom et al., 2000; Marchi et al., 2002; Pidoplichko et al., 2004). Hence, the cumulative result of nicotine acting on nAChRs in the VTA is enhanced excitatory input onto DA neurons which triggers reward-related high frequency burst firing resulting in increased accumbal DA outflow (Corrigall et al., 1994; Nisell et al., 1994; Schilstrom et al., 2000; Pidoplichko et al., 2004).

Several labs have investigated the specific subunit compositions of the nAChRs that may be critical in the development of nicotine dependence using cell-based heterologous expression systems, transgenic mouse lines, and pharmacological manipulations using nAChR ligands in animal behavior models (Tapper et al., 2004; Steensland et al., 2007; Chatterjee and Bartlett, 2010; Cahir et al., 2011). The α4β2* and α6β2* nAChR subtypes play an essential role in mediating the rewarding effects of nicotine (Dani and De Biasi, 2001; Nashmi et al., 2007), demonstrated by a lack of nicotine-elicited DA release in β2 and α4 knockout mice and a decrease in nicotine self-administration by mice lacking the α4, α6, or β2 subunits compared to wild-types (Picciotto et al., 1998; Marubio et al., 2003; Pons et al., 2008). By contrast, the role α7 nAChRs play in the reinforcing properties of nicotine is less clear. Deletion of the α7 did not affect nicotine conditioned place preference (Walters et al., 2006) or self-administration (Pons et al., 2008); however, high doses of the α7 nAChR antagonist methyllycaconitine attenuated nicotine self-administration (Markou and Paterson, 2001) and reduced the rewarding effects of nicotine when infused directly into the VTA (Laviolette and van der Kooy, 2003).

The effects of ethanol in the VTA are more complex than the effects of nicotine. Ethanol, unlike nicotine, is not a direct agonist at nAChRs (Cardoso et al., 1999; Zuo et al., 2002) but can modulate DA release by influencing the function of nAChRs in both the VTA and NAc (Ericson et al., 1998, 2003; Larsson et al., 2005). While local perfusion of ethanol into the VTA does not increase DA release in the accumbens, infusion of ethanol into the accumbens does elevate extracellular DA to a similar degree as systemic administration (Ericson et al., 2003; Tuomainen et al., 2003). However, ethanol infused into both regions simultaneously resulted in higher DA levels than when injected into the NAc alone (Lof et al., 2007). Furthermore, perfusion of mecamylamine (MEC, non-selective nAChR antagonist) into the VTA, but not into the NAc, blocks DA release stimulated by systemic administration of ethanol. It has been postulated that ethanol's actions in the NAc may facilitate the release of endogenous ACh in the VTA, leading to activation of nAChRs and consequently elevating accumbal DA release (Larsson et al., 2005). In support, voluntary ethanol intake in rats has been shown to cause

a significant increase in ACh levels in the VTA which is time-locked with the DA increase in the NAc (Larsson et al., 2005). It should be mentioned here that in addition to nAChRs, ethanol is known to modulate DA neurotransmission through actions at other molecular targets [for review (Vengeliene et al., 2008)]. For example, ethanol (10–80 mM) acting on pre-synaptic D_1 receptors can increase excitatory glutamate transmission in the VTA and enhance DA release (Xiao et al., 2009).

While, it is relatively clear that α4β2* and α7 nAChRs play a major role for nicotine mediated dopamine effects in the VTA, it has been more difficult to determine the specific nAChR compositions important for ethanol. Several subunit-specific antagonists have been administered to mice both systemically and by direct infusion into the brain. Ethanol-induced DA release in the NAc and locomotor activity were blocked by systemic injections of MEC but not by methyllycaconitine citrate (MLA, α7 antagonist) or dihydro-β-erythroidine (DHβE, broad-spectrum β2*-antagonist), suggesting ethanol's stimulatory effects may not be mediated by β2*-containing or α7 nAChRs (Larsson et al., 2002). Another study further defined which subunits were involved in ethanol's effects on the DAergic system by administering various α-conotoxins into the VTA and measuring DA outflow in the NAc following an ethanol challenge. Results showed that α-conotoxin MII, selective for α3β2* and/or β3* and/or α6* subunits, significantly reduced ethanol-stimulated DA release in the NAc, while the selective α6* antagonist, α-conotoxin PIA-analog, showed no effect (Larsson and Engel, 2004; Jerlhag et al., 2006). Taken together, this evidence suggests ethanol's actions in the VTA are mediated by α3β2* and/or β3*, rather than α4β2*, α7, or α6* nAChRs; however, it remains unclear if these effects are due to direct or indirect interactions of ethanol with nAChRs.

nAChR-mediated synaptic plasticity. In addition to augmenting transmitter release, drugs of abuse can induce long-lasting plasticity within midbrain DA centers following both acute and chronic drug administration (Gao et al., 2010). In the VTA, drug-evoked NMDA-mediated plasticity may occur in a similar fashion to that seen in the hippocampus and requires activation of pre-synaptic voltage-gated ion channels. Indeed, several drugs of abuse can elicit LTP in VTA excitatory synapses and increase AMPA receptors without changing the number or function of NMDA receptors (Saal et al., 2003; Jin et al., 2011). Activation of nAChRs by nicotine likely influence the persistent potentiation of these excitatory synapses (Jin et al., 2011), however at this time it is unclear if and how nAChRs play a role in ethanol-mediated NMDAR-dependent plasticity.

There is experimental evidence illustrating nicotine-induced plasticity (**Table 1**) in VTA slice preparations where application of nicotine (500 nM) was shown to increase the AMPA/NMDA receptor ratio by increasing pre-synaptic release of glutamate. This effect is mediated by α7 nAChRs since pre-incubation with the α7 antagonist (MLA), but not the β2*-antagonist (DHβE), abolished the nicotine-induced LTP. In support, α7 knockout mice lack this response to nicotine (Jin et al., 2011). Furthermore, the nicotine-induced enhancement of excitatory currents lasts after prolonged exposure suggesting a lack of α7 desensitization (Pidoplichko et al., 2004) and persists even after nicotine is removed from the bath, thereby indicating LTP (Mansvelder and McGehee, 2000; Dani, 2001; Mansvelder et al., 2002).

Striatum

The striatum is a heterogeneous structure, with distinct anatomical and functional subterritories that can be broadly classified into the dorsal and ventral striatum. The majority (>90%) of the neurons in the striatum are GABAergic medium spiny neurons (MSN) with most (∼80%) of the synapses being asymmetric glutamatergic inputs (Wilson, 2007; Tepper et al., 2008). In addition, there are at least three other types of interneurons, including a small population of cholinergic interneurons (∼2%) that provide a rather extensive arborization in this region to modulate DA release probability (Zhou et al., 2002; Pakhotin and Bracci, 2007). The dopaminergic neurons arising from the VTA project to the ventral striatum (NAc and part of the olfactory tubercle), while the dorsal striatum (caudate-putamen) receives dopaminergic inputs primarily from the substantia niagra pars compacta. The ventral striatum is highly involved in the reinforcing effects of drugs of abuse and receives extensive excitatory afferents from the PFC, amygdala and hippocampus (Carelli, 2002; Volkow et al., 2006). On the other hand, efferent projections from the motor cortex to the dorsal striatum allows this subregion to gate sensorimotor function and have been implicated in the advanced stages of habitual drug seeking (Fasano and Brambilla, 2002; Gerdeman et al., 2003; Philibin et al., 2011).

There is a limited number of subunits (α4, α5, α6, β2, and β3) densely localized on pre-synaptic DAergic axon terminals in the striatum (Grady et al., 2007), where β2-containing nAChRs can directly influence local DA release based on the neuronal activity firing rate (see **Figure 2**) (Zoli et al., 2002; Rice and Cragg, 2004). In the proposed model, ACh released from tonically active striatal cholinergic interneurons normally acts on these receptors to gate the probability of DA release and enhance the contrast between tonic and phasic firing patterns. In a manner similar to those in the VTA, these β2-nAChRs will rapidly desensitize following agonist application. Initial nicotine administration causes a reduction of dopamine release from tonically active neurons; however, a reward-related burst of action potentials will result in even greater transmitter release due to loss of the normal modulatory control of endogenous Ach (Zhou et al., 2001; Rice and Cragg, 2004; Salminen et al., 2004; Zhang and Sulzer, 2004).

Besides anatomical and connectivity differences, there are clear distinctions in DA signaling between the ventral and dorsal striatum which govern their specific brain functions and behavioral output. For example, initial drug use will favor DA release in the NAc shell rather than the dorsolateral striatum (Pontieri et al., 1996; Di Chiara, 2002). Furthermore, these two regions respond differently to stimulus trains that mimic action potentials, with tonic and phasic firing eliciting greater DA release from the dorsolateral striatum and NAc shell, respectively (Zhang et al., 2009). Recently, it was demonstrated that activity-dependent DA transmission in the ventral striatum is mainly controlled by α6*-containing nAChRs (α6α4β2β3 and α6β2*) but in the dorsal striatum non-α6 nAChRs (α4β2 and α4α5β2) are the key players (Exley et al., 2007, 2008, 2011). In summary, DA release in the striatum is modulated by different nAChRs on pre-synaptic

DAergic terminals in a frequency-dependent and region-specific manner.

nAChR-mediated synaptic plasticity. In the NAc, synapses can express both LTP and LTD (Kombian and Malenka, 1994) but mainly NMDAR-dependent LTD has been demonstrated following administration of psychostimulants, such as cocaine and amphetamine, which involves the endocytosis of AMPARs. Although in some situations ethanol has been shown to inhibit NMDAR activity and associated plasticity (Blitzer et al., 1990), activation of DA D1 receptors will diminish ethanol's inhibition of NMDARs thus promoting reinforcement and plasticity in the NAc (Maldve et al., 2002). Ethanol treatment (50 mM) has been shown to cause a short-term depression of the striatal output and this effect is sensitive to nAChR antagonists, MEC (10 μM) and MLA (40 nM), which block the depression of synaptic output (Adermark et al., 2011).

Neuronal morphological changes, including increases in spine density or dendritic length, have been reported following environmental enrichment or drug administration (Johansson and Belichenko, 2002; Leggio et al., 2005) and are thought to reflect structural reorganization of neural circuits manifested by molecular events discussed thus far in this review. In the NAc, long-lasting structural plasticity has been demonstrated in MSN following nicotine administration; however, these changes may be dependent on treatment schedule and cohort age. One study found an increase in dendritic length and spine density in the NAc and PFC of adult rats after intermittent subcutaneous injections of nicotine (Brown and Kolb, 2001) while another reported significant increases in dendritic length and branch number in the NAc shell of adolescent but not adult rats after continuous nicotine administration (**Table 1**) (McDonald et al., 2007).

Prefrontal cortex

The PFC is a key brain region regulating executive cognitive function, attention, and working memory. Dysregulation of normal signaling in the PFC, paralleled by deficits in cognitive performance, have been observed in disorders such as Alzheimer's disease, schizophrenia, ADHD, and Parkinson's disease (Picciotto and Zoli, 2002). Interestingly, nicotine has been shown to enhance cognition and attention in people suffering from these disorders (Rezvani and Levin, 2001). The PFC has extensive connections to reward and memory hubs, receiving DA innervations from the VTA and providing glutamatergic efferents to the VTA and NAc (Sesack et al., 1989; Carr and Sesack, 2000). It is now well established that drugs of abuse may take over the normal operations of this system, driving impulsivity and compulsive behaviors characteristic of addiction (Lasseter et al., 2010). Recent neuroimaging studies of PFC activity in drug-addicted subjects point to global dysfunction in this region that is associated with a greater incidence of relapse and heavier drug use (Goldstein and Volkow, 2011).

Similar to other brain regions, both α7 and non-α7 nAChR signaling pathways converge in the PFC to modulate both excitatory and inhibitory neurotransmission (see **Figure 2**). Although glutamatergic pyramidal cells in layer V lack nAChRs, nicotine increases EPSCs and Glutamate release from thalamo-cortical afferents that do express them (Gioanni et al., 1999; Lambe et al., 2003; Couey et al., 2007). There are two populations of GABAergic interneurons, fast spiking and non-fast spiking, with only the latter bearing nAChRs (α7 and α4β2*) (Gabbott et al., 1997; Kawaguchi and Kondo, 2002). Activation of both α7 and β2-containing nAChRs on glutamatergic nerve endings enhance the release of excitatory amino acids but do so by different mechanisms (Dickinson et al., 2008). α7 nAChRs, predominately found on ryanodine positive terminals, mediate release by calcium-induced calcium release (CICR) which is coupled to the activation of pre-synaptic extracellular signal-regulated kinase (ERK2) and phosphorylation of synapsin-1. Non-α7 nAChRs recruit voltage-gated calcium channels to induce release of [^{3}H] D-aspartate (Dickinson et al., 2008). Additionally, β2-containing nAChRs govern glutamatergic neurotransmission and activation of this receptor subtype by nicotine enhances glutamate release onto layer 5 and 6 pyramidal neurons (Gioanni et al., 1999; Lambe et al., 2003).

nAChR-mediated synaptic plasticity. DA has been shown to be a strong modulator of synaptic plasticity in the PFC by fine tuning glutamatergic transmission and facilitating the induction of LTP or LTD (Otani et al., 2003; Matsuda et al., 2006). Systemic and local administration of nicotine will enhance DA overflow in the medial PFC of rodents (Nisell et al., 1996; Marshall et al., 1997) and both β2-containing and α7 nAChRs influence this response (**Table 1**) (Livingstone et al., 2009). In rat PFC slices, DA acting through D1 and D2 receptors consistently resulted in LTD and required postsynaptic depolarization and Ca^{2+} influx, but was independent of NMDAR activation (Law-Tho et al., 1995; Otani et al., 1998). In contrast, *in vivo* stimulation of the VTA (250 Hz) induced LTP in hippocampo-PFC projections through cooperative actions of D1 and NMDA receptors (Gurden et al., 1999, 2000).

In the PFC, the relative timing of action potentials in pre- and post-synaptic neurons is critically important for determining the direction of synaptic plasticity, either LTP or LTD, and is referred to as spike-timing-dependent plasticity (STDP) (Markram et al., 1997; Bi and Poo, 1998; Couey et al., 2007). When the pre-synaptic spike occurs before the post-synaptic spike in a time-sensitive manner, robust LTP is induced; in contrast, reversing the order of stimulation will result in LTD. Nicotine will increase the threshold for induction of STDP under the same stimulus conditions by reducing dendritic calcium signals that normally occur with action potential propagation. Pyramidal neurons in the PFC lack nAChRs but GABAergic inhibitory control of these cells is modulated by nicotinic receptors (Couey et al., 2007). The ability of nicotine to eliminate the induction of LTP is attributed to activation of nAChRs on GABA interneurons and glutamatergic cells, which both ultimately increase the excitation of GABA interneurons and enhance inhibition of layer V pyramidal neurons. Application of a $GABA_A$ receptor antagonist or increasing dendritic calcium signals with burst-like, post-synaptic stimulation blocked nicotine's effects and produced STDP similar to that of control conditions. The authors speculate that one way nicotine may improve cognition is by increasing the signal-to-noise ratio during PFC neural processing (Couey et al., 2007).

Table 1 | nAChRs modulate synaptic transmission in the mesolimbic system.

Location	nAChR subtype	Agonist	Outcome	Mechanism	References
Ventral tegmental area	α7 on presynaptic glutamatergic neurons	Nicotine	1. ↑ Dopamine (DA) release in the nucleus accumbens 2. Promotes long-term potentiation (LTP)	1. ↑ glutamate release onto NMDARs located on DAergic cell bodies 2. ↑ frequency of spontaneous postsynaptic currents (sEPSCs)	Mansvelder and McGehee, 2000; Schilstrom et al., 2000; Pidoplichko et al., 2004
Nucleus accumbens		Nicotine	↑ dendritic length and branches		Brown and Kolb, 2001; McDonald et al., 2007
Prefrontal cortex	Activation of nAChRs on soma of GABAergic interneurons	Nicotine	↑ threshold for induction of spike-timing-dependent plasticity	1. ↑ GABAergic inputs to PFC layer 5 pyramidal neurons 2. ↑ inhibitory postsynaptic currents (IPSCs) 3. Reduces post-synaptic Ca^{2+} signals	Couey et al., 2007
	Activation of nAChRs on glutamatergic terminals	Nicotine	↑ threshold for induction of spike-timing-dependent plasticity	1. ↑ glutamate release onto fast spiking interneurons 2. ↑ GABAergic inputs to PFC layer 5 pyramidal neurons 3. ↑ IPSCs 4. Reduces post-synaptic Ca^{2+} signals	Couey et al., 2007
		Nicotine	↑ dendritic length and branches		Brown and Kolb, 2001

AMYGDALA AND HIPPOCAMPAL COMPLEX: LEARNING AND MEMORY

Nicotinic receptors have long been known to play a significant role in cognition and disruption of normal nAChR function has been demonstrated in diseases such as Alzheimer's and schizophrenia (Paterson and Nordberg, 2000). nAChR agonists, including nicotine, enhance cognition, memory and promote learning by actions in a number of different brain circuits; however, the underlying mechanisms are still not completely understood (Levin et al., 1994; Socci et al., 1995; Levin and Simon, 1998). An important aspect of addiction is the development of context-drug associations and the formation of memories that link drug-predictive cues to the reinforcing properties of the substance. Studies in both animals and humans have highlighted the importance of associated learning of drug intake with both environmental and internal cues in mediating future drug-seeking and relapse (Fuchs et al., 2008).

The hippocampus and amygdala have been implicated in mediating some of the cognitive-enhancing effects of nicotine, supported by findings that show microinfusion of nAChR agonists can enhance memory-related functions, while antagonists impair them (Ohno et al., 1993; Felix and Levin, 1997). For example, direct infusion of nicotine into basolateral nucleus of the amygdala enhanced working memory and facilitated the acquisition and consolidation of short- and long-term memories; on the other hand, infusion of MLA had opposite effects on memory performance (Barros et al., 2005). Microinfusion of DHβE or MLA into basolateral nucleus of rats resulted in working memory deficits in the radial-arm maze, indicating both α7 and α4β2* nAChRs in this area are involved in normal memory function (Addy et al., 2003). In addition, nicotine dose-dependently stimulates the release of norepinephrine in the amygdala and hippocampus by activating nAChRs localized on norepinephrinergic neurons in the brainstem (Fu et al., 1998). Norepinephrine contributes to memory function and the stress response (Liang et al., 1990; Bremner et al., 1996), offering yet another mechanism by which nicotine modulates these processes.

Few studies have reported the effects of ethanol on nAChRs in these areas. One study reported that co-administration of ethanol (i.p.) and nicotine infusions into the CA1 region or basolateral nucleus of the amygdala produced a significant conditioned place preference, while either drug alone did not; furthermore, this response was blocked by microinjection of mecamylamine into the CA1 or basolateral nucleus of the amygdala (Zarrindast et al., 2010). Taken together, nAChRs in the amygdala and hippocampus play a prominent role in not only learning and memory but also reward-related learning.

Hippocampus

From an anatomical prospective, the hippocampus is integrally linked to brain circuits involved in addiction, receiving direct dopaminergic input from midbrain neurons and providing extensive efferent connections to the ventral striatum,

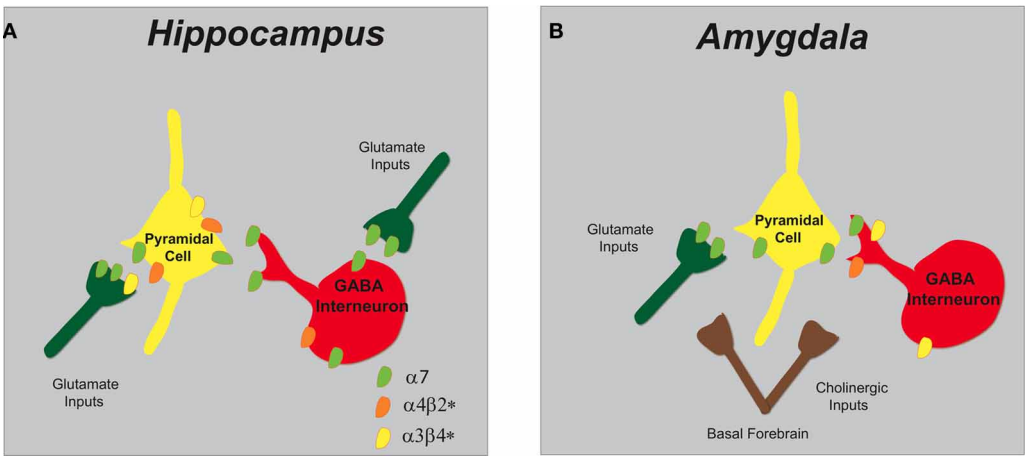

FIGURE 3 | Schematic representation of nAChR subtypes and circuit function in the hippocampus and amygdala. (A) In the hippocampus, α7 and α4β2* nAChRs are abundantly expressed on pyramidal cells and inhibitory interneurons. GABAergic interneurons have pre-synaptic α7 nAChRs and somato-dendritic expression of α7 and α4β2* nAChRs. Glutamatergic afferents have predominately pre-synaptic α7 nAChRs and only low levels of α3β4*. **(B)** In the amygdala, cholinergic inputs from the basal forebrain synapse in proximity to pre-synaptic nAChRs that modulate both excitatory and inhibitory synaptic transmission. Glutamatergic afferents and pyramidal neurons endow α7 nAChRs and GABAergic interneurons express multiple nAChRs (α7, α4β2*, and α3β4*).

amygdala, and PFC (Kelley, 2004). Therefore, alterations in structure or function in the hippocampus may be translated by other brain regions that drive maladaptive behaviors associated with addiction. Most studies investigating the involvement of nAChRs in synaptic plasticity have been conducted in the hippocampus.

The α7 and α4β2* nAChRs (see **Figure 3**) are abundantly expressed on GABAergic interneurons and pyramidal cells within the hippocampus and are capable of modulating intracellular signaling molecules and downstream effectors that govern plasticity (Jones and Yakel, 1997; Vizi and Lendvai, 1999). GABAergic interneuron populations express α7 nAChRs on pre-synaptic terminals, whereas somato-dendritic compartments endow both α7 and α4β2* nicotinic receptors (Radcliffe et al., 1999; Alkondon and Albuquerque, 2001). Furthermore, modulation of glutamate synaptic transmission to pyramidal neurons in the CA1 region is attributed to predominately α7 nAChRs but also to a minimal number of α3β4* nAChRs (Gray et al., 1996; Ji et al., 2001; Alkondon and Albuquerque, 2002).

nAChR-mediated synaptic plasticity. Nicotinic receptors exert a temporally- and spatially-dependent bidirectional control over synaptic plasticity, both *in vitro* and *in vivo* (**Table 2**). For example, in the CA1 region of hippocampal slices ACh and nicotine can act on post-synaptic receptors of pyramidal neurons to increase intracellular Ca^{2+} which facilitates the conversion of short-term potentiation to LTP by reducing the threshold needed for induction (Fujii et al., 1999; Ji and Dani, 2000; Nakauchi et al., 2007) or by attenuating the inhibitory input of interneurons to pyramidal cells (Ji and Dani, 2000; Yamazaki et al., 2005); these effects are mediated by both the activation of non-α7 nAChRs and the inactivation of α7 nAChRs (Fujii et al., 2000a). Furthermore, blunting the evoked release of inhibitory GABA onto pyramidal cells to facilitate nicotine-induced LTP induction was shown to rely on desensitization of non-α7 nAChRs (Fujii et al., 2000b; Yamazaki et al., 2005; Nakauchi et al., 2007). Additionally, activation of nAChRs on hippocampal interneurons can induce LTP or LTD depending on the exact timing of agonist application in respect to the pre-synaptic stimulation (Ji et al., 2001; Ge and Dani, 2005). Furthermore, activation of α7 nAChRs on pre-synaptic glutamatergic terminals can increase the frequency of miniature EPSCs and enhance glutamate release onto pyramidal neurons offering yet another mechanism for the modulation of plasticity (Gray et al., 1996; Radcliffe and Dani, 1998). In the CA3 region of hippocampal slices, bath application of nicotine can drive the pyramidal cells above threshold in the absence of an action potential by activating pre-synaptic nAChRs located on glutamatergic terminals. Activation of these receptors enhances miniature EPSCs and glutamate release through mobilization of intracellular calcium stores by CICR (Sharma and Vijayaraghavan, 2003).

Within the dentate gyrus, induction of LTP by nicotine required activation of mGLuR5 and L-type Ca^{2+} channels, as well as Ca^{2+} release from ryanodine-sensitive stores and was α7 nAChR-dependent (Welsby et al., 2006, 2009). *in vivo* studies in mice showed nicotine or epibatidine, an α4β2 nAChR agonist, dose-dependently induced synaptic plasticity in the dentate gyrus and importantly, required intact midbrain DA signaling (Matsuyama et al., 2000; Matsuyama and Matsumoto, 2003; Tang and Dani, 2009). In the developing brain, long-lasting changes in synaptic transmission were observed following a single exposure to nicotine in the hippocampus. nAChR signaling facilitated the conversion of pre-synaptic silent synapses into functional ones and was shown to be dependent on α7 nAChRs most likely

localized on pre-synaptic glutamatergic nerve endings (Maggi et al., 2003). Together these findings strongly imply that the timing and location of nAChR activity are important determinants for synaptic plasticity in the hippocampus.

Amygdala

The amygdala is another essential brain region implicated in memory processing, particularly for encoding the emotional and motivational significance of environmental stimuli as well as initiating innate unconditioned responses to aversive situations. It is a central region for integrating sensory and cognitive information through its extensive connections to other limbic structures, the cortex, hippocampus, and thalamus (LeDoux, 1996). In addition, experimental evidence strongly suggests drugs of abuse act on this system and can modify synaptic events, especially during periods of withdrawal (McCool et al., 2010).

Reciprocal connections between the amygdala, hypothalamus and parabrachial nucleus are known to regulate the hypothalamic-pituitary-adrenal axis and autonomic responses to conditioned fear (Takeuchi et al., 1982; Gray et al., 1989). The amygdala also participates in stress- and reward-related behaviors through its connections to the PFC and NAc, respectively (Simpson et al., 2001; Myers-Schulz and Koenigs, 2012; Stuber et al., 2011). The basolateral nucleus of the amygdala is densely innervated by cholinergic projections arising from the basal forebrain (Sah et al., 2003), with cholinergic inputs synapsing on pyramidal neurons (89% of efferents) and GABAergic interneurons (Muller et al., 2011). Different pathways reside within the amygdala and are responsible for various functions regarding the acquisition, expression, and retrieval of fear memories as well as unconditioned behaviors (LeDoux, 2003).

Functional nAChRs expressed on pyramidal cells (somatodendritic α7), GABAergic interneurons (α7, α4β2*, and α3β4*), and glutamatergic afferents (α7) modulate synaptic transmission in the amygdala (see **Figure 3**) (Hill et al., 1993; Perry et al., 2002; Klein and Yakel, 2006). Whole cell patch-clamp recordings demonstrated nicotine increases the frequency of both glutamatergic and GABAergic spontaneous post synaptic currents (PSCs) and this effect was sensitive to the α7-selective antagonist α-bungarotoxin, thus implicating a role of pre-synaptic α7 nAChRs (Barazangi and Role, 2001). Another report showed that activation of predominately α3β4* nAChRs on GABAergic interneurons in the basolateral nucleus of the amygdala were responsible for the enhanced frequency of inhibitory PSCs (Zhu et al., 2005).

nAChR-mediated synaptic plasticity. In the amygdala (**Table 2**), nicotine has been shown to facilitate LTP in a pathway-specific manner. Robust LTP in amygdala slices from mice that received nicotine treatment for 7 days compared to controls and persisted 72 h after nicotine cessation. Even just one day of nicotine exposure significantly enhanced LTP. The nicotine-induced facilitation of LTP was found to be dependent on both α7 and β2-containing nAChRs and at least partially, to activation of nAChRs on GABAergic interneurons that ultimately reduce inhibition of pyramidal neurons. Furthermore, LTP was completely blocked by D-APV, an NMDAR antagonist, demonstrating the essential role of NMDARs in nicotine-mediated plasticity in the amygdala. The authors suggest that as seen in other brain regions, nicotine may also be acting on pre-synaptic nAChRs on glutamatergic terminals to enhance glutamate release or increasing postsynaptic Ca^{2+} influx through voltage-dependent calcium channels (Huang et al., 2008). At this time, little is known about nicotinic receptor-mediated plasticity in the amygdala. Ethanol is capable of modulating synaptic changes in this circuit but it has yet to be elucidated if and how nicotinic receptors are involved.

CHANGES IN nAChRs NUMBER AND FUNCTION

NICOTINE

A primary mechanism underlying long-lasting synaptic plasticity is a change in the number or expression of membrane-bound receptors. Long-term exposure to nicotine induces an up-regulation of specific subtypes of nAChRs and increases the number of high-affinity nicotinic binding sites across multiple brain regions in the brains of postmortem human smokers (Perry et al., 1999) and nicotine-treated rodents (Schwartz and Kellar, 1983; Flores et al., 1992; Marks et al., 1992; Gentry and Lukas, 2002). The concept of up-regulation of nAChRs is somewhat unexpected and contradictory to what the homeostatic model would predict. Following chronic drug use, receptors are usually down regulated in response to excessive stimulation as an adaptive mechanism to adjust the neural network to a pre-exposure point. Evidence suggests that nicotine causes a rapid desensitization of nAChRs, and this loss in receptor function would promote up-regulation to compensate for the diminished signaling of inactivated receptors over prolonged periods of time (Fenster et al., 1999a,b). These changes result in higher sensitivity to nicotine and have been correlated with nicotine addiction [see review, Govind et al. (2009)].

Several mechanisms have been proposed for nicotine-induced up-regulation of nAChRs and it is quite likely that more than one mechanism is responsible for this phenomenon. There is controversy surrounding how this up-regulation of surface receptors occurs but it does not appear to be due to a change in subunit mRNA transcript levels (Marks et al., 1992; Bencherif et al., 1995; Ke et al., 1998) but has been proposed to be caused by increased translation or alterations in receptor assembly (Wang et al., 1998; Nashmi et al., 2003), trafficking (Harkness and Millar, 2002), and/or decreased receptor turnover (Wang et al., 1998). For example, nicotine has been shown to inhibit the turnover of cell-surface receptors of the α4β2* conformation. The authors, using cell line M10, demonstate that up-regulation of α4β2* nAChRs is due to an intrinsic property of these proteins and results from a conformational change of the receptors that makes their degradation and removal from the cell surface slower (Peng et al., 1994). Another possible mechanism is an increase in receptor trafficking to the cell surface upon long exposures to nicotine (Harkness and Millar, 2002). An increase in the intracellular receptor pool caused by enhanced receptor assembly and/or maturation of the subunits in the endoplasmic reticulum has also been proposed (Nashmi et al., 2003). Additionally, nicotine can reportedly facilitate receptor maturation by acting as a chaperone in the endoplasmic reticulum (Nashmi et al., 2003; Srinivasan et al., 2011). However, membrane-impermanent

Table 2 | nAChRs modulate synaptic plasticity in the hippocampus and amygdala.

Location	nAChR subtypes	Agonist	Outcome	Mechanism	References
Hippocampus CA1 region	Post-synaptic activation of non-α7 and inactivation of α7	Nicotine (acute/chronic) and ACh	1. Reduces threshold for long-term potentiation (LTP) 2. Converts short-term potentiation to LTP	1. ↑ intracellular Ca^{2+} in pyramidal neurons 2. ↑ neuronal excitability	Fujii et al., 1999, 2000a; Ji and Dani, 2000; Nakauchi et al., 2007
	Desensitization of non-α7 on pre-synaptic GABAergic interneurons	Nicotine	Promotes LTP induction	Disinhibition of pyramidal neurons	Ji and Dani, 2000; Fujii et al., 2000b; Yamazaki et al., 2005
	Activation α7 on glutamatergic nerve terminals	Nicotine and ACh	Silent synapses to functional	Facilitation of synaptic transmission	Maggi et al., 2003
	α7 on presynaptic glutamatergic neurons	Nicotine	Promotes LTP induction	1. Ca^{2+} influx—excitatory post-synaptic currents (EPSCs) 2. ↑ glutamate release onto pyramidal neurons	Gray et al., 1996; Radcliffe and Dani, 1998
	Dependent on type and location of nAChRs	ACh	LTP or LTD induction	Timing of postsynaptic nAChR activation and pre-synaptic stimulation	Ji et al., 2001; Maggi et al., 2004; Ge and Dani, 2005
Hippocampus CA3 region	nAChRs activation on glutamatergic neurons	Nicotine	Brings post-synaptic pyramidal neurons to action potential threshold	1. Ca^{2+} influx-EPSCs 2. Ca^{2+} induced Ca^{2+} release 3. ↑ glutamate release onto pyramidal neurons	Sharma and Vijayaraghavan, 2003
Dentate gyrus	α7	Nicotine	Enhance tetanus-induced LTP	1. Depedent on NMDAR and voltage-activated Ca^{2+}channels 2. ryanodine-sensitive Ca^{2+} stores 3. Ca^{2+} induced Ca^{2+} release	Welsby et al., 2006, 2009
	α7 and α4β2*	Nicotine, epibatidine, choline	LTP induction	Requires dopamine input	Matsuyama et al., 2000; Matsuyama and Matsumoto, 2003; Tang and Dani, 2009
Amygdala	α7 and β2-containing	Nicotine *(in vivo* exposure)	Facilitate LTP	1. NMDAR-dependent 2. Reduces inhibition of pyramidal neurons	Huang et al., 2008

ligands can also induce up-regulation of surface receptors; therefore, second messengers must exist that are sufficient to drive this response (Whiteaker et al., 1998; Darsow et al., 2005). In order for nicotine-induced up-regulation to occur, nAChRs must pass through the secretory pathway before being inserted into the membrane (Darsow et al., 2005) suggesting that up-regulation is not due to stabilization of nAChRs in the plasma membrane.

The up-regulation of nAChRs varies with subunit composition, cell type and brain region. Amongst the different subtypes of nAChRs, studies have shown the α4β2*, α3β2*, and α6β2* nAChRs can be activated, desensitized and up-regulated by nicotine concentrations (peak levels between 100 and 500 nM) achieved following cigarette smoking in humans (Nguyen et al., 2003; Parker et al., 2004). The α7 and β4-containing nAChRs appear to be less sensitive to nicotine and require higher concentrations ($<10\,\mu M$) to activate them. The two accessory subunits α5 and β3 appear to inhibit nicotine-induced receptor up-regulation or down-regulation. In this respect, no up-regulation was reported when α5 subunits were associated with α4β2 nAChRs (Mao et al., 2008) and in the striatum, α6-containing receptors without β3 were down-regulated by nicotine while those containing β3 were unaffected (Perry et al., 2007).

ETHANOL

There are a limited number of studies that have investigated ethanol-induced changes in expression of nAChRs and therefore it is certainly an area of research which should be expounded upon. *In vitro* experiments demonstrated nAChRs are directly affected by ethanol and after long-term exposure these receptors may undergo anatomical and functional changes, possibly by altering receptor expression or composition (Dohrman and Reiter, 2003). In M10 cells, ethanol modulates the number of nAChRs by initially blunting the expression during short exposure (6–72 h) but increasing it with longer incubation periods (96 h). Similar results were found with co-application of relatively high concentrations of nicotine (1 μM) and ethanol (100 mM); moreover, the elevated receptor expression after chronic exposure (96 h) remained up to 7 days following the removal of the drugs (Dohrman and Reiter, 2003). In a different study, long-term consumption of ethanol (5 months) by rats increased the levels of [^{3}H]-nicotinic binding in the hypothalamus and thalamus, and decreased the levels in the hippocampus (Yoshida et al., 1982). In ethanol-treated (6 months) mice, small changes in [^{3}H]-nicotinic binding were found only in the thalamus and in just one of the mice strains tested, leading the authors to conclude this effect is brain region specific and genetic factors may influence this response (Booker and Collins, 1997). These effects were not seen in mouse brains following short-term (1–2 weeks) ethanol treatment (Burch et al., 1988; de Fiebre and Collins, 1993).

Finally, receptor up-regulation should enhance neuronal excitability and favor induction of drug-induced LTP. Thus, it can be hypothesized that drug exposure leads to a chain reaction of interrelated events: up-regulation of nAChRs—LTP/LTD—enhanced neurotransmitter release—behavioral modifications, which will all contribute to uncontrollable drug use (Vezina, 2004).

BEHAVIORAL IMPLICATIONS OF PLASTICITY

One behavioral correlate of synaptic plasticity is the manifestation of locomotor sensitization, which is defined as an enhanced locomotor response after repeated exposures to a drug compared to the activity measured during the first drug administration. Increased locomotor response to prolonged nicotine, ethanol, cocaine, amphetamine, and methamphetamine has been extensively studied in rodent animal models and is thought to have relevance to drug seeking and relapse in humans (Steketee and Kalivas, 2011).

Data suggests repeated administration of a drug causes altered dopaminergic and glutamatergic transmission in the mesocorticolimbic system (Vanderschuren and Kalivas, 2000; Pascual et al., 2009) and is associated with the neuroplastic changes discussed thus far in this review. Up-regulation of receptors may not be the sole cause of drug-induced locomotor sensitization, since the timing of these events don't necessarily correlate, but likely plays a role in the development of this behavioral response (Vezina, 2007). nAChRs are up-regulated in the entire brain after long exposures to nicotine but nAChRs in the VTA and NAc are the most probable regions implicated for the induction of sensitization (Parker et al., 2004). Long-lasting behavioral sensitization has been shown to correlate well with LTP, reflecting persistent adaptations in neural mechanisms such as the modulation of synaptic strengths, change in neurotransmitter release, alterations in gene expression and formation of new connections between synapses. In the next section, we will focus on nicotine and ethanol's effect on behavioral sensitization.

NICOTINE AND ETHANOL STIMULATED LOCOMOTOR SENSITIZATION

Several studies have shown nicotine induces locomotor sensitization in mice and rats by a range of nicotine doses (0.1–2 mg/kg, i.p) and by different schedules of administration (Domino, 2001; Collins and Izenwasser, 2004; Saito et al., 2005). Typically, the first nicotine injection produces locomotor depression which is rapidly overcome by subsequent nicotine exposure and is associated with the development of tolerance to the drug's acute depressant effect (Morrison and Stephenson, 1972). This enhanced locomotor activity in response to repeated nicotine administration is long-lasting (Miller et al., 2001) and central nicotinic receptors play a key role. In support, mecamylamine (Bevins and Besheer, 2001), lobeline (non-selective nAChR antagonists) (Miller et al., 2003), and SSR591813 (α4β2 partial agonist) (Cohen et al., 2003) all blocked induction of sensitization by nicotine. In a separate study, pre-treatment with mecamylamine but not α-bungarotoxin (α7 nAChR antagonist) prevented sensitization, implicating non-α7 nAChRs in mediating the locomotor-stimulant effects of nicotine (Kempsill and Pratt, 2000).

While nicotine–induced sensitization has been widely studied, motor stimulant effects of ethanol have generally received less attention. The development of sensitization to ethanol is predominantly shown in mice. Similar to nicotine-induced locomotor activity, mice were pre-treated with ethanol injections (1.5–2.5 g/kg, i.p) for 7–10 days. Following this exposure, they were challenged with a single injection of ethanol after a period of withdrawal (7–30 days). Results indicated the mice were significantly more sensitive to the locomotor stimulating effects of ethanol during this challenge session and this effect lasted up to 29 days following termination of ethanol administration (Lessov and Phillips, 1998; Itzhak and Martin, 2000; Fish et al., 2002). Under similar circumstances, stimulation of locomotor activity by ethanol consuming rats has also been reported (Hoshaw and Lewis, 2001).

There is a substantial amount of evidence supporting the idea that activation of the DAergic system is required for the emergence of the sensitized locomotor response, with induction of sensitization attributed to the VTA and the expression to the NAc (Mao and McGehee, 2010). Through actions on nAChRs in this system, both nicotine and ethanol influence neuronal activity firing rate (Mereu et al., 1987), bursting activity (Zhang and Sulzer, 2004), and corresponding neurotransmitter release—including DA, GABA, and Glutamate, which are surely contributing to the drugs' locomotor-stimulating effects (Nestby et al., 1997; Guo et al., 1998; Tzschentke, 2001; Lambe et al., 2003; Broadbent et al., 2005; Meyer et al., 2008; Mao et al., 2011). For example, intracranial injections of nicotine directly into the VTA results in locomotor sensitization (Reavill and Stolerman, 1990; Kita et al., 1992) and is associated with an increase in DA and c-Fos-like immunoreactivity (an indicator of neuronal activation) in the

NAc (Panagis et al., 1996; Shim et al., 2001). For these reasons, behavioral sensitization induced by nicotine and ethanol can be partially attributed to their actions on nAChRs in the midbrain reward pathway.

CROSS SENSITIZATION

While repeated exposure to a single drug can produce behavioral sensitization, sometimes cross-sensitization between drugs is observed. In this type of experiment, animals are repetitively treated with a particular drug for a period of time and then challenged with a different drug after a defined drug-free period. Although the animal has experienced a different drug, locomotor sensitivity to the challenge drug is observed, indicating a common molecular substrate. For example, caffeine, cocaine, and amphetamine have all been shown to produce cross-sensitization to nicotine-induced hyperlocomotion (Collins and Izenwasser, 2004; Celik et al., 2006; Santos et al., 2009). Others studies have demonstrated cocaine and ethanol exhibit cross-sensitization of locomotor effects (Itzhak and Martin, 1999). The findings for nicotine and ethanol are mixed, with some studies reporting no cross-sensitization (Watson and Little, 1999; Darbra et al., 2004) while others report observing this phenomenon (Biala and Weglinska, 2004; Biala and Budzynska, 2010). There are, however, other behavioral measures that clearly illustrate a common molecular interaction between these two substances. In this respect, rats with prior exposure to nicotine show increased ethanol consumption (Blomqvist et al., 1996); furthermore, ethanol-induced locomotor activity and accumbal DA release is blocked by mecamylamine, indicating a pivotal role of nAChRs in ethanol's behavioral effects (Blomqvist et al., 1992; Larsson et al., 2002). In addition, drugs acting through nAChRs, including a partial agonist (varenicline) and non-selective antagonist (MEC), reduce ethanol consumption in both rodents and humans (Le et al., 2000; Steensland et al., 2007; McKee et al., 2009).

CONCLUSIONS

This review has summarized multiple different mechanisms that underlie persistent, long-lasting changes in synaptic efficacy following administration of addictive drugs. It is becoming more and more evident that nicotinic receptors significantly facilitate the induction and maintenance of plasticity—including LTP, LTD, and structural changes—in the hippocampus, amygdala, and mesolimbic dopaminergic system, thus contributing to the molecular underpinnings of nicotine and alcohol addiction. Nicotine exerts its powerful effects by a dynamic, parallel activation, and desensitization of nAChRs. Up-regulation of nAChRs following nicotine treatment reflects a compensatory response to excessive receptor stimulation, and there is compelling experimental evidence to suggest this plays a major part in nicotine dependence. Although few studies have addressed ethanol-induced synaptic plasticity via interactions with nicotinic receptors, ethanol undoubtedly potentiates nAChR currents and drugs targeting nAChRs can attenuate voluntary alcohol consumption in both rodents and humans. Importantly, there is a need to understand the molecular and cellular ramifications of co-administration of nicotine and ethanol due to the high comorbidity of these substances in human addicts. Future studies should aim to unravel the common neural mechanisms shared by these two drugs. This review has touched upon the behavioral outcomes of repeated administration of drugs of abuse, thus suggesting that long-lasting changes in synaptic strength and modification of neurotransmitter release contribute to locomotor sensitization. Both nicotine and ethanol alone clearly induce behavioral sensitization, and cross-sensitization may or may not occur between these two substances. At this time, a large body of literature exists regarding the mechanism of action of nicotine but there is still much to be elucidated pertaining to ethanol's actions at nAChRs for synaptic plasticity and behavioral sensitization.

Clearly, nicotine can enhance cognitive function and propagate LTP and thereby these processes are likely, at least in part, what underlie the highly addictive nature of this compound. Reports from human users of cognitive deficits and strong cue-induced cravings during nicotine withdrawal undoubtedly contribute to the high incidence of relapse. Medications that target neural substrates directly involved in both learning and addiction may offer a novel pharmacotherapeutic approach for nicotine dependence as well as other drugs of abuse. More intriguing yet is the possibility that novel therapeutic avenues may be directed to diminish drug-associated memories or facilitate the formation of new memories with less maladaptive behavioral consequences.

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Neuroplasticity in addiction: cellular and transcriptional perspectives

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Drug addiction is a chronic, relapsing brain disorder which consists of compulsive patterns of drug-seeking and taking that occurs at the expense of other activities. The transition from casual to compulsive drug use and the enduring propensity to relapse is thought to be underpinned by long-lasting neuroadaptations in specific brain circuitry, analogous to those that underlie long-term memory formation. Research spanning the last two decades has made great progress in identifying cellular and molecular mechanisms that contribute to drug-induced changes in plasticity and behavior. Alterations in synaptic transmission within the mesocorticolimbic and corticostriatal pathways, and changes in the transcriptional potential of cells by epigenetic mechanisms are two important means by which drugs of abuse can induce lasting changes in behavior. In this review we provide a summary of more recent research that has furthered our understanding of drug-induced neuroplastic changes both at the level of the synapse, and on a transcriptional level, and how these changes may relate to the human disease of addiction.

Keywords: addiction, plasticity, CREB, deltaFosB, epigenetics, histone modification, DNA methylation, microRNAs

INTRODUCTION

Drug addiction is a chronic, relapsing disorder characterized by uncontrolled, compulsive drug use that persists despite serious negative consequences. One of the most insidious features of addiction is the enduring susceptibility to relapse displayed by users despite months or even years of abstinence (O'Brien, 1997). Importantly, not everyone who uses drugs becomes addicted, and whether or not a person makes this transition can be influenced by a complex interplay of genetic and environmental factors (Goldman et al., 2005; Kendler et al., 2007). The escalation of drug use from casual to compulsive and the persistent vulnerability to relapse is thought to be underpinned by long-lasting neuroadaptations in brain reward circuits (Thomas et al., 2008; Luscher and Malenka, 2011; Robison and Nestler, 2011). Essentially all drugs of abuse exert their acute reinforcing properties via the mesocorticolimbic dopamine pathway, encompassing dopamine neurons that originate in the ventral tegmental area (VTA) and project to the striatum and other limbic regions including the prefrontal cortex (PFC), amygdala and hippocampus (Di Chiara and Imperato, 1988; Le Moal and Simon, 1991). The striatum also receives glutamatergic input from the PFC, and while mesolimbic dopamine is no doubt important for the initial stages of drug-taking and reinforcement, a role for corticostriatal glutamate transmission in the compulsive and enduring nature of addiction is being increasingly recognized (Kalivas, 2009; Kalivas et al., 2009). A major focus of research at present lies in characterizing the cellular and molecular changes that occur within this motivational circuitry to contribute to the development and persistence of addiction. In the laboratory, various behavioral facets of addiction can be investigated using animal models (summarized in **Table 1**). The purpose of this review is to provide an overview of the neuroplastic changes that occur both at the synapse, and on the level of gene transcription, that contribute to addiction-related behaviors.

SYNAPTIC PLASTICITY MECHANISMS: ADDICTION AS A PATHOLOGICAL FORM OF LEARNING AND MEMORY

The observation that drug-taking and relapse are quite often directly linked to exposure to drug-related cues highlights the importance of associative learning mechanisms in addiction (Wikler and Pescor, 1967; Tiffany and Drobes, 1990; O'Brien et al., 1998). Steven Hyman made the point that "memory disorders are often thought of as conditions involving memory loss, but what if the brain remembers too much or too powerfully records pathological associations?" (Hyman, 2005). In this context, addiction can be perceived, at least in part, as a pathological form of learning and memory. In support of this hypothesis research over the last decade has demonstrated that drugs of abuse do indeed modify synaptic plasticity in the mesocorticolimbic and corticostriatal circuitry by similar mechanisms that underlie long-term memory formation. What these modifications actually represent in terms of behavior and addiction more generally is another, perhaps more challenging, question. The following section will overview the synaptic adaptations caused by drugs of abuse as measured electrophysiologically in the context of animal models and their relevance to the addicted state.

It was Santiago Ramon y Cajal who, over 100 years ago, contemplated the idea that changes in the strength of synaptic

Table 1 | Modeling addiction in animals.

Locomotor sensitization: Locomotor sensitization describes the progressive increase in locomotor activity that usually follows repeated, intermittent drug exposure. Sensitization can persist for months or even years following withdrawal, and as such it is considered to be an indication of enduring drug-induced plasticity (Steketee, 2003). Although it is most commonly studied in relation to psychostimulants, sensitization has also been characterized in response to opiates, nicotine and ethanol (Shuster et al., 1977; Kalivas and Duffy, 1987; Robinson et al., 1988; Benwell and Balfour, 1992; Cunningham and Noble, 1992). Cross-sensitization between different drugs of abuse has also been shown to exist, suggesting that common mechanisms underlie the development of this phenomenon despite these drugs having distinct pharmacological actions in the brain (Vezina and Stewart, 1990; Itzhak and Martin, 1999; Beyer et al., 2001; Cadoni et al., 2001).

Conditioned place preference (CPP): CPP is an indirect measure of drug reward based on classical (Pavlovian) conditioning principles (Tzschentke, 1998). The CPP apparatus consists of two distinct environments, one of which is paired with a drug, and with repeated pairing the drug-paired environment acquires secondary motivational properties which can elicit approach behavior. An animal is said to have obtained a place preference if it spends more time in the drug-paired environment when given a choice. This paradigm is used to measure conditioned drug reward and associative learning.

Operant self-administration: Animals can be trained to self-administer most drugs that are commonly abused by humans. This is usually achieved using operant boxes where an instrumental task such as a lever press or nose poke results in the delivery of a drug or natural reward. Reward delivery can be paired with a discrete cue such as a tone or light, or passive contextual cues.

Extinction/reinstatement: Extinction describes a reduction in conditioned drug-seeking behavior after it is repeatedly non-reinforced (Myers and Davis, 2002). Extinction can be performed in the context of CPP, where an animal is repeatedly exposed to the drug-paired environment in the absence of the drug. Once a CPP is extinguished, it can be reinstated by drug priming (Mueller and Stewart, 2000) or exposure to stressors (Sanchez and Sorg, 2001; Wang et al., 2006). Operant self-administration behavior can also be extinguished by removal of drug reinforcement, and subsequently reinstated by non-contingent exposure to the drug (Dewit and Stewart, 1981), exposure to cues or contexts previously associated with the drug (Meil and See, 1996; Weiss et al., 2000; Crombag and Shaham, 2002), or exposure to stress (Shaham and Stewart, 1995; Erb et al., 1996; Shepard et al., 2004). These same factors are known to precipitate drug craving and relapse in human addicts, and as such reinstatement attempts to model relapse-like behavior in animals.

connections between neurons could be the way in which the brain stores information (Cajal, 1894). The discovery of long-term potentiation (LTP) in the hippocampus in 1973 provided the first evidence that this may be the case (Bliss and Lomo, 1973). LTP is the enhancement of synaptic strength that results from synchronous firing of connecting neurons, whereas its counterpart long-term depression (LTD) is the weakening of synaptic strength (Citri and Malenka, 2008). These processes usually involve N-methyl-D-aspartate (NMDA) receptor-mediated trafficking of α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) receptors to and from the cell surface (Kauer and Malenka, 2007). An NMDA receptor-mediated increase in calcium levels in the postsynaptic cell is required for the induction of LTP and LTD, with the amount of calcium determining the sequence of events. Large increases in calcium preferentially activate protein kinases and result in LTP, ultimately expressed as enhanced transmission at postsynaptic AMPA receptors. In contrast, more modest increases in calcium preferentially activate protein phosphatases and produce LTD, which is expressed as a decrease in AMPA receptor transmission (Kauer and Malenka, 2007). While LTP and LTD were initially studied in relation to learning and memory in the hippocampus, they are now known to occur at most excitatory synapses throughout the central nervous system, and are important for many forms of experience-dependent plasticity (Malenka and Bear, 2004; Kauer and Malenka, 2007).

DRUG-EVOKED POTENTIATION AT EXCITATORY SYNAPSES IN THE VTA

A pioneering study by Ungless and colleagues in 2001 demonstrated that a single exposure to cocaine caused an enhancement of synaptic strength at excitatory synapses on VTA DA neurons when measured 24 h later in brain slices (Ungless et al., 2001). This was measured as an increase in the ratio of AMPA-mediated excitatory postsynaptic currents (EPSCs) over NMDA-mediated EPSCs (termed the AMPA/NMDA ratio). Subsequent electrically-evoked LTP was shown to be occluded at excitatory VTA synapses in cocaine-treated mice whereas LTD was enhanced. These observations as well as a number of other electrophysiological measures indicated that the change in plasticity observed potentially shared similar mechanisms to synaptically-evoked LTP (Ungless et al., 2001). It has since been shown that administration of other drugs of abuse including amphetamine, morphine, ethanol, nicotine, and benzodiazepines can also induce increases in synaptic strength in the VTA, an effect that is not seen with psychoactive drugs that do not have abuse potential (Saal et al., 2003; Gao et al., 2010; Tan et al., 2010). This observation demonstrates a convergence of cellular responses within the VTA by all abused drugs and provides a possible neural mechanism by which initial neuroadaptations underlying addiction could be triggered.

The effect of non-contingent drug administration on VTA synaptic plasticity is transiently expressed, lasting at least 5 but less than 10 days and has been shown to positively correlate with the initial development of behavioral sensitization but not with its expression (Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004). If cocaine is self-administered the outcome is rather different as plasticity in the VTA becomes persistent and can be detected even 90 days into withdrawal (Chen et al., 2008). The potentiation of glutamatergic synapses on VTA DA cells is presumably linked to the ability of drugs of abuse to enhance extracellular DA in the NAc (Di Chiara and Imperato, 1988) and potentially represents the initiation of "pathological" reward

learning whereby a "stamping in" of drug-cue associations occurs. Indeed, NMDA receptor-dependent increases in glutamatergic synaptic strength have been reported in VTA DA neurons during the acquisition of a cue-reward association (Stuber et al., 2008) and recently it was confirmed that cocaine selectively increases the AMPA/NMDA ratio of VTA neurons which project to the NAc as opposed to the PFC (Lammel et al., 2011); it is well established that dopamine transmission within the NAc is critical for the acquisition of a Pavlovian association (Kelley, 2004). Thus it may be that that potentiation of VTA DA neurons may represent neural coding similar to LTP, possibly an associative learning process, which may be essential for early cocaine-induced behavioral responses and has the capacity to trigger long-term adaptations that underlie addiction, though does not represent the addicted state itself. As proposed by others, it may be that addictive drugs co-opt brain reward circuitry to "overlearn" the value of a drug to the organism (Kauer and Malenka, 2007).

The origins of the pertinent glutamatergic projections to the VTA involved in drug-induced plasticity remain to be fully elucidated. One study has revealed that VTA glutamatergic synapses targeted by projections from both the VTA itself and the pedunculopontine nucleus (PPN) show enhanced potentiation from cocaine yet only the synapses receiving input from PPN afferents are potentiated with Δ^9-tetrahydrocannabinol (THC) (Good and Lupica, 2010). Thus, it appears that the particular glutamatergic afferents involved in drug-induced potentiation can vary according to the drug in question and it may also be the case that a particular projection is common to all drug-evoked excitatory plasticity in the VTA; the latter is yet to be determined. The VTA receives extensive projections from multiple brain regions including the PFC, amygdala and subthalamic nucleus (Geisler and Wise, 2008), many of which have been shown to influence the burst firing of VTA DA neurons (Grillner and Mercuri, 2002). Future experiments utilizing optogenetic techniques could assist in determining the particular projections responsible the drug-evoked potentiation at VTA synapses observed in response to various drugs of abuse, thus shedding light on the on the exact nature of this neuroadaptation.

MECHANISMS UNDERLYING DRUG-EVOKED SYNAPTIC PLASTICITY AT EXCITATORY SYNAPSE IN THE VTA

As with electrically-induced LTP in midbrain DA neurons the increase in synaptic strength in the VTA induced by both cocaine and nicotine has been shown to be dependent on NMDA receptor activation (Bonci and Malenka, 1999; Ungless et al., 2001; Mao et al., 2011). In contrast, the maintenance of cocaine-evoked potentiation was recently shown to require the activity of protein kinase Mζ (Ho et al., 2012), an autonomously active protein kinase C (PKC) isoform, whereas spike timing-dependent LTP in VTA DA neurons of drug-naïve mice depends on conventional PKC isoforms (Luu and Malenka, 2008). In the case of nicotine the VTA synaptic potentiation requires the excitation of DA neurons mediated by somatodendritic α4β2 nicotinic acetylcholine receptors (nAChRs) (Mao et al., 2011). Nicotine-induced increases of presynaptic glutamate release also contribute to the induction of this particular synaptic plasticity, likely through increased activation of NMDA receptors (Mao et al., 2011).

Relatively more is known about the mechanisms underlying cocaine-evoked synaptic plasticity than that underlying plasticity induced by other drugs of abuse. Cocaine application to midbrain slices results in potentiation of NMDA receptor transmission within minutes and is proposed to be via insertion of NR2B-containing NMDARs into synapses through a mechanism that requires activation of D_5 receptors and new protein synthesis (Schilstrom et al., 2006; Argilli et al., 2008). Orexin A has also been shown to be required for both rapid cocaine-induced insertion of NR2B-containing receptors and increased AMPA/NMDA ratios; accordingly the orexin$_1$ receptor antagonist SB334867 has been shown to prevent the development of sensitization to cocaine (Borgland et al., 2006). In addition to changes in NMDA receptor subunit expression, increased levels of GluR1-containing (GluR2-lacking) AMPA receptors at synapses have been observed as soon as 3 h after cocaine exposure (Argilli et al., 2008). This observation combined with other recent evidence has led to the hypothesis that synaptic insertion of high-conducting GluR2-lacking receptors contribute to expression of cocaine-induced synaptic potentiation in the VTA (Dong et al., 2004; Bellone and Luscher, 2006; Mameli et al., 2007; Brown et al., 2010; Mameli et al., 2011), for reviews see (Kauer and Malenka, 2007; Wolf and Tseng, 2012). This insertion of GluR2-lacking AMPA receptors depends on NMDA receptor transmission in VTA DA neurons since it is absent in mice lacking functional NMDA receptors in DA neurons (Engblom et al., 2008; Mameli et al., 2009). Insertion of GluR2-lacking AMPA receptors is significant because they have unique properties; they are calcium permeable, have greater single channel conductance than GluR2-containing receptors, and therefore have a huge capacity to alter synaptic transmission (Isaac et al., 2007). Hence, insertion of GluR2-lacking AMPA receptors in the VTA represents a possible mechanism by which drugs of abuse can instantiate the plastic adaptations underlying the initial stages of drug use.

The insertion of GluR2-lacking AMPA receptors into VTA excitatory synapses has now been shown to occur in response to administration of drugs from multiple classes such as nicotine and morphine as well as upon optogenetic activation of DA VTA neurons (Brown et al., 2010). This has led to the proposal that insertion of calcium-permeable GluR2-lacking AMPA receptors represents a universal mechanism which may underlie drug-evoked potentiation of VTA synapses (Brown et al., 2010), though the data for amphetamine are not necessarily consistent with this hypothesis (Faleiro et al., 2004). Moreover, as GluR2-lacking AMPA receptors are inwardly rectifying and thus conduct very little current at +40 mV, their insertion alone cannot explain drug-evoked increases in AMPA/NMDA ratios. A recent study which measured unitary synaptic responses evoked by a highly localized glutamate source (two-photon photolysis of caged glutamate) showed, than in addition to affecting AMPA receptor-mediated EPSCs, cocaine exposure also decreased unitary NMDA receptor-mediated EPSCs (Mameli et al., 2011), thus providing a possible mechanism by which AMPA/NMDA ratios could be increased in this scenario (by lowering the denominator of the ratio). This is yet to be investigated with other drugs of abuse.

The drug-induced exchange of GluR2-containing with GluR2-lacking AMPA receptors can be reversed by activation of mGluR1 receptors in the VTA (Bellone and Luscher, 2006; Mameli et al., 2007). Thus, mGluR1-mediated exchange of AMPA receptors provides a mechanism which can explain why drug-evoked potentiation of VTA synapses is transient in nature, lasting 5 but not 10 days (Ungless et al., 2001; Mameli et al., 2007). Indeed, if mGluR1 function in the VTA is reduced 24 h before cocaine administration then cocaine-induced inward rectification persists beyond 7 days (Mameli et al., 2007, 2009). Hence one possible explanation for why cocaine-evoked synaptic strengthening persists in the VTA following self-administration of cocaine (unlike following non-contingent administration) could be that cocaine self-administration leads to depression of mGluR1 signaling in the VTA.

DRUG–EVOKED SYNAPTIC PLASTICITY AT INHIBITORY SYNAPSES IN THE VTA

Excitatory synapses are not the only type of synapse in VTA DA neurons which are affected by non-contingent administration of drugs of abuse. Inhibitory synapses in the VTA also have a critical role in controlling the firing rate of DA neurons, thus plasticity at GABAergic synapses has the capacity to dramatically influence DA transmission. Indeed, cocaine, morphine and ethanol can all influence inhibitory synaptic plasticity in the VTA (Melis et al., 2002; Liu et al., 2005; Nugent et al., 2007). Repeated cocaine exposure *in vivo* for 5–7 days causes a reduction in amplitudes of GABA-mediated synaptic currents, thereby facilitating LTP induction in VTA cells by reducing the strength of GABAergic inhibition (Liu et al., 2005). Subsequent studies reveal the mechanism of this inhibition to be endocannabinoid-dependent LTD at GABAergic synapses involving activation of ERK1/2 (Pan et al., 2008, 2011). $GABA_A$ receptor synapses on VTA dopamine neurons also exhibit robust NMDA-dependent LTP (termed LTP_{GABA}) in response to high-frequency stimulation (Nugent et al., 2007). This LTP_{GABA} is absent in VTA slices 2 and/or 24 h after *in vivo* administration of morphine, nicotine, cocaine or ethanol (Nugent et al., 2007; Guan and Ye, 2010; Niehaus et al., 2010). In the case of ethanol the prevention of LTP_{GABA} is mediated by the μ-opioid receptor (Guan and Ye, 2010) Together with synaptic potentiation at excitatory synapses, this loss of LTP_{GABA} should increase the firing of VTA DA neurons following drug exposure.

Slow GABA transmission has also recently been shown to be affected by drugs of abuse. Thus a single dose of methamphetamine or cocaine is sufficient to significantly weaken the ability of $GABA_B$ receptors to control VTA GABA neuron firing when measured *ex vivo* 24 h later (Padgett et al., 2012). The methamphetamine-induced loss of the slow inhibitory post-synaptic potential (IPSC) arises from a reduction in $GABA_B$ receptor-G protein-coupled inwardly-rectifying potassium channel (GIRK) currents, due to changes in protein trafficking, and is accompanied by a significant decrease in the sensitivity of presynaptic $GABA_B$ receptors in GABA neurons of the VTA. Unlike drug-induced influences on $GABA_A$ synapses this depression of $GABA_B$R-GIRK signaling persists for days after the injection (Padgett et al., 2012).

BEHAVIOURAL CORRELATES OF DRUG-EVOKED POTENTIATION IN VTA DA CELLS

As mentioned earlier the effect of non-contingent drug administration on synaptic plasticity in VTA DA neurons is transiently expressed, lasting at least 5 but less than 10 days and has been shown to positively correlate with the initial development of behavioral sensitization but not with its expression (Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004). In support of the hypothesis that drug-evoked potentiation of VTA synapses represents induction of behavioral sensitization, intra-VTA administration of glutamate antagonists reduce, and virally-mediated GluR1 up-regulation enhances the locomotor sensitizing properties of drugs (Carlezon et al., 1997; Carlezon and Nestler, 2002). Strong evidence of NR2A- and B-containing NMDA receptor involvement is provided by the observation that pharmacological inhibition of either prevents both the development of sensitization and associated cocaine-induced increases in AMPA/NMDA ratios (Schumann et al., 2009). However, mice with targeted deletion of NR1 or GluR1 (selective to midbrain DA neurons) or global GluR1 deletion exhibit intact behavioral sensitization and yet show impaired AMPA receptor currents after cocaine treatment (Dong et al., 2004; Engblom et al., 2008). An added twist is provided by the observation that CPP and conditioned locomotor behavior is absent in GluR1 knockout mice (Dong et al., 2004) and extinction of cocaine CPP is absent in mice with GluR1 deletion targeted to midbrain DA neurons (Engblom et al., 2008), whereas in NR1 knockout mice reinstatement of cocaine CPP and expression of behavioral sensitization is attenuated (Engblom et al., 2008; Zweifel et al., 2008). Thus, even with the caveat of potential developmental compensation in mutant mice and/or possible incomplete deletion, it is possible that that neural processes governing drug-evoked potentiation of DA neurons and behavioral sensitization are dissociated. Rather it may be that potentiation of VTA synapses may contribute to the attribution of incentive salience to drug-associated cues.

Measuring synaptic changes following non-contingent drug administration is limited with respect to informing on the actual disease state of addiction. More relevant to the human condition are studies where changes in synaptic plasticity are measured following contingent drug administration e.g., operant self-administration. In this regard, synaptic strengthening of VTA DA cells induced by self-administration of cocaine is uniquely persistent, lasting 3 months into abstinence and shown to be resistant to extinction training (Chen et al., 2008). Thus, though initially proposed to be a transient event, it appears that drug-evoked plasticity in the VTA has the capacity to be long-lasting, demonstrating that the method of administration (contingent versus non-contingent) is a critical determinant of its longevity. This is supported by the observation that yoked controls in this study did not show a similar increase in AMPA/NMDA ratio; suggesting it is the learning of the cue-reward or action-outcome association which is driving plasticity. In contrast, self-administration of food or sucrose under similar parameters produce increases in AMPA/NMDA ratios that persist for 7 but not 21 days into abstinence, demonstrably transient compared to that induced by cocaine (Chen et al., 2008). The lack

of persistence of food-induced plasticity demonstrates that the change in synaptic strength induced by cocaine is not merely a neural representation of the instrumental or cue-reward learning processes involved in the operant self-administration paradigm *per se*, rather a drug-specific effect which potentially represents a pathological strengthening of drug-cue associations. As mentioned previously, cues predicting reward have also been found to cause an increase in AMPA/NMDA ratios in the VTA, though not as persistent, supporting a role for this modification of excitatory synaptic function in reward learning (Stuber et al., 2008).

Interestingly, the magnitude of the increase in the AMPA/NMDA ratio is similar regardless of the number of injections (single vs. multiple), administration protocol (contingent vs. non-contingent), and length of access (limited access vs. extended access) (Borgland et al., 2004; Chen et al., 2008; Mameli et al., 2009). This indicates that the increase in AMPA/NMDA ratio observed in VTA DA cells is potentially a permissive event, perhaps signaling "salience" as opposed to representing an initiation of underlying neuropathology which would presumably increase with continued exposure.

DRUG-EVOKED PLASTICITY AT EXCITATORY SYNAPSES IN THE NAc

Unlike the VTA a single cocaine injection does not cause increases in synaptic strength in the NAc when measured 24 h later (Thomas et al., 2001; Kourrich et al., 2007). This observation and the bidirectional timescale which follows with repeated administration and withdrawal demonstrates that drug-induced plasticity in the NAc is markedly different from that observed in the VTA. Indeed, when repeated injections of cocaine are administered (so as to induce behavioral sensitization), a decrease in the AMPA/NMDA ratio is observed at NAc shell synapses when measured 24 h after the last administration (Kourrich et al., 2007). This synaptic depression from repeated cocaine appears to be linked to plasticity in the VTA; upon selective disruption of mGluR1 function in the VTA only a single injection of cocaine is then required to cause this same depression of NAc synapses (Mameli et al., 2009). The authors of this study postulate that enhanced excitation of VTA projections may facilitate the coincident release of DA and glutamate in the NAc through an enhanced release of DA. This may then shift the threshold for the induction of local plasticity in the NAc by affecting circuit excitability or by integrating intracellular signaling processes (Mameli et al., 2009).

The functional significance of the depression of NAc synapses during acute withdrawal is unclear at this stage. One possible explanation may be that depression of NAc medium spiny neurons (MSNs) reduces their response to natural rewarding stimuli, hence contributing to the anhedonia experienced during acute withdrawal. It could also be that the decrease observed in the AMPA/NMDA ratio may be result of membrane insertion of NR2B-containing NMDA receptors (thus increasing the denominator of the ratio) as new silent synapses are found to occur in the NAc shell upon cocaine exposure (Huang et al., 2009). Silent glutamatergic synapses, which express functional NMDA receptor-mediated currents in the absence of AMPA receptor-mediated currents, are thought to possess an increased capacity to undergo strengthening of synaptic transmission (Isaac et al., 1995). Once generated, these silent synapses may facilitate recruitment of AMPA receptors thereby enhancing excitatory synaptic transmission. This provides a possible mechanism to explain increases in the surface level of AMPA receptors and subsequent AMPAR/NMDAR ratio observed in the NAc during protracted withdrawal (Boudreau and Wolf, 2005; Boudreau et al., 2007; Kourrich et al., 2007; Conrad et al., 2008). NR2B-containing NMDA receptors in the NAc could also been involved in the formation of drug-context associations as siRNA knockdown of this subunit prevents morphine CPP in mice but not behavioral sensitization (Kao et al., 2011).

Unlike cocaine, a repeated regimen of intermittent ethanol exposure results in a potentiation of synapses in response to a previously LTD-inducing stimulation protocol when measured 24 h after the last exposure (Jeanes et al., 2011). This NMDA-dependent potentiation is transient as after a further 48 h of withdrawal it has dissipated and neither LTP nor LTD can be induced (Jeanes et al., 2011). The authors interpret such robust changes in NAc plasticity as an indicator of the potential importance of this process in ethanol-induced neuroadaptations. Moreover, unlike psychostimulants, ethanol can act at NMDA receptors so therefore has the capacity to directly influence glutamatergic signaling.

SYNAPTIC POTENTIATION OBSERVED IN THE NAc AFTER A PERIOD OF WITHDRAWAL

In contrast to the depression observed during acute withdrawal, potentiation of NAc shell synapses is observed after 10–14 days of withdrawal from repeated cocaine or morphine administration (Kourrich et al., 2007; Wu et al., 2012). Moreover, after 7 days withdrawal from a single administration of cocaine, an increase in the amplitude of mEPSCs as well as a loss of LTP induced by high frequency stimulation (HFS) is found in both core and shell NAc neurons expressing the dopamine D_1 receptor (Pascoli et al., 2012). This change in the ability to induce synaptic plasticity is referred to as metaplasticity. Cocaine-induced metaplasticity is also observed following withdrawal from cocaine self-administration. Thus, rats that have self-administered cocaine followed by 3 weeks of either extinction or abstinence display a marked *in vivo* deficit in the ability to develop LTP in the NAc core after stimulation of the PFC. This observation was accompanied by a leftward shift in the input-output curve suggesting potentiation of fEPSP amplitude (Moussawi et al., 2009). Potentiation of NAc synapses is also observed in the form of increased AMPA-mediated currents following an extended period of abstinence after self-administration (Conrad et al., 2008). Collectively, these data suggest that synaptic potentiation in the NAc develops either as a function of duration of withdrawal, or as a function of time since the first administration of cocaine. A recent study supports the latter interpretation as similar increases in the frequency of mEPSCs was observed in D_1 receptor-expressing MSNs in mice despite the absence or presence of a protracted withdrawal period following repeated cocaine administration (Dobi et al., 2011). Therefore, it seems the events leading to the changes in glutamatergic transmission in the NAc take some time to develop.

The contribution of specific AMPA receptor subunits to this change varies according to the stage of withdrawal and the method of administration; 10–21 days into withdrawal from both passive and self-administration GluR2-containing AMPA receptors appear to be responsible for changes in AMPA transmission (Boudreau and Wolf, 2005; Boudreau et al., 2007; Kourrich et al., 2007; Ferrario et al., 2010) whereas beyond 21 days GluR2-lacking AMPA receptors are added to synapses. The latter finding appears to be the case only when cocaine is self-administered (Conrad et al., 2008; McCutcheon et al., 2011), though see (Mameli et al., 2009). Given the increased conductance of GluR2-lacking AMPA receptors it may be that their insertion occurs in response to the depression of NAc synapses caused by cocaine self-administration, thereby resulting in increased MSN responsiveness to excitatory inputs that trigger cocaine-seeking in the future. Indeed, blocking GluR2-lacking AMPA receptors in the NAc prevents expression of incubated cue-induced cocaine seeking (Conrad et al., 2008), and cocaine-seeking induced by either AMPA or cocaine is also blocked by injections of antisense oligonucleotides of GluR1 mRNA into the NAc (Ping et al., 2008).

DRUG CHALLENGE AFTER WITHDRAWAL REVERTS SYNAPTIC POTENTIATION TO DEPRESSION

The increase in synaptic strength and surface expression of AMPA receptor subunits induced by cocaine in the NAc after withdrawal from non-contingent administration is subsequently reversed upon administration of further cocaine injections (re-challenge) (Thomas et al., 2001; Boudreau et al., 2007; Kourrich et al., 2007; Ferrario et al., 2010). Thus, synaptic depression is once again observed in the NAc shell when measured 24 h after this cocaine injection (Thomas et al., 2001), though see (Pascoli et al., 2012). Behaviorally this appears to correlate with the expression of sensitization, and in the case of amphetamine at least, has been shown to be clathrin-mediated and reliant on GluR2-dependent endocytosis of postsynaptic AMPA receptors (Brebner et al., 2005). The decrease in surface expression of AMPA receptors following cocaine challenge is transient as within 7 days surface expression recovers to levels comparable to unchallenged cocaine-pretreated rats (Ferrario et al., 2010). As such, it appears that history of cocaine exposure and withdrawal can readily change the direction of synaptic plasticity in the NAc.

A direct link was recently made between the potentiation of cortico-accumbal synapses on D_1 receptor-positive cells following 7 days withdrawal and the expression of sensitization. As mentioned previously, after 7 days withdrawal from a single administration of cocaine, these synapses are found to be potentiated in both the core and shell (as measured by an increase in mEPSC amplitude) and LTP induced by HFS is reduced. The same was not found for synapses on D_2 receptor-positive cells (Pascoli et al., 2012). When reversed optogenetically *in vivo* via a protocol known to induce LTD, cortico-accumbal synapses on D_1-receptor positive cells displayed reduced mEPSCs and the expression of locomotor sensitization was prevented. Importantly, the ability of HFS to induce LTP was restored to these neurons (Pascoli et al., 2012), thus demonstrating a direct link between this particular synaptic adaptation at cortico-accumbal synapses and the expression of sensitization to cocaine.

PERSISTENT IMPAIRMENTS IN NAc CORE PLASTICITY UNDERLIE THE TRANSITION TO ADDICTION

As mentioned above, it appears that cocaine induces metaplastic changes in NAc MSNs. The term "metaplasticity" was originally coined by Abraham and Bear to describe the change in the ability of synapses to undergo future plasticity (Abraham and Bear, 1996). Thus, a loss of LTD is observed in both the NAc core and shell 24 h following the end of cocaine self-administration; however after 21 days abstinence this deficit is found exclusively in the core (Martin et al., 2006). The same deficit is not found in yoked animals nor animals that have self-administered food, demonstrating it to be specific to the voluntary self-administration of cocaine and not associated with instrumental learning nor the cocaine exposure *per se* (Martin et al., 2006), thus raising the possibility that drug-induced metaplasticity in the NAc core may underlie the transition from casual use to compulsive drug-seeking behavior. The impairment in NAc synapses induced by cocaine self-administration may manifest in drug addicts as an inability to inhibit their behavior and thus prevent compulsive drug-intake.

Subsequent *in vivo* electrophysiological experiments support this hypothesis. Self-administered cocaine followed by extinction training was shown to induce metaplasticity which impaired the ability of PFC stimulation to produce LTP or LTD in NAc core MSNs (Moussawi et al., 2009). Moreover, administration of N-acetylcysteine, a drug which normalizes glutamate levels and reduces craving in addicts (Amen et al., 2011), was found to reverse this cocaine-induced metaplasticity and restore the ability to induce LTP or LTD (Moussawi et al., 2009). These findings have been extended to an animal model of relapse, the reinstatement model (see **Table 1**). Treatment with N-acetylcysteine was shown to attenuate reinstatement of drug-seeking induced by either cue or prime, an effect that persisted 2 weeks beyond cessation of treatment. Importantly, this attenuation was linked to its ability to restore synaptic strength to cortico-accumbal synapses (Moussawi et al., 2011).

These data provide a possible causal relationship between cocaine-induced plasticity at cortico-accumbal synapses and susceptibility to relapse, consistent with a glutamate homeostasis theory of addiction. Thus, a failure of the PFC to control drug-seeking behaviors can be linked to an enduring imbalance between synaptic and non-synaptic glutamate (Kalivas, 2009). Chronic cocaine results in reduced basal levels of glutamate due to down-regulation of the cystine-glutamate exchanger. This removes tone from presynaptic mGlu2/3 receptors located at cortico-striatal synapses which normally function to limit glutamate release (Kalivas, 2009). N-acetylcysteine inhibits drug-seeking by activating the cystine-glutamate exchanger, thereby increasing extrasynaptic glutamate and stimulating presynaptic mGluR2/3 receptors to reduce the glutamate release associated with drug-seeking (Kalivas, 2009). Given the strong link between mGluR2/3 regulation of both synaptic glutamate release and drug-seeking, the capacity of mGluR2/3 antagonist to inhibit N-acetylcysteine restoration of LTP is consistent with the possibility that normalizing cortico-accumbal plasticity is ameliorative in terms of relapse (Moussawi et al., 2009).

Further evidence supporting a key role for adaptations at NAc glutamatergic synapses in drug-seeking behavior is provided by observations that up-regulation of GluR2-lacking AMPA receptors mediate the incubation of cocaine craving seen after extended abstinence from cocaine (Conrad et al., 2008), and disrupting trafficking of GluR2-containing AMPA receptors in either the NAc core or shell attenuates the capacity of cocaine to reinstate extinguished drug-seeking behavior (Famous et al., 2008). Enhanced AMPA receptor-mediated transmission appears to be particularly relevant to drug-seeking. Thus, intra-NAc core administration of an AMPA receptor agonist promotes while an antagonist inhibits cocaine-seeking (Cornish and Kalivas, 2000) and similar results are found for both heroin (Lalumiere and Kalivas, 2008) and alcohol (Backstrom and Hyytia, 2004). Indeed, increased AMPA-mediated transmission is consistent with a critical role for prefrontal glutamate release NAc core in mediating reinstatement of drug-seeking behavior (McFarland et al., 2003; Kalivas et al., 2005).

Given this established role for increased AMPA-mediated glutamate in drug-seeking behavior, is potentially not surprising that primed reinstatement of heroin-seeking in rats was recently shown to require LTP-like increases in synaptic strength at cortico-accumbal synapses (Shen et al., 2011). This increase in synaptic strength was accompanied by changes in spine remodeling and required up-regulation of the NR2B subunit of the NMDA receptor (Shen et al., 2011). Further studies examining synaptic potentiation as a result of drug-seeking in the absence of a drug prime will provide insight into the exact synaptic changes elicited by the drug-seeking behavior itself.

By examining synaptic changes in the context of models of chronic self-administration and drug-seeking behavior following extinction or abstinence, it is more likely that experimental outcomes will reflect the changes occurring in the brains of drug addicts as opposed to the being the result of drug exposure alone. Nevertheless, while it is apparent that drug self-administration induces long-lasting changes in synaptic transmission, it is unknown whether these are non-specific adaptations that occur in all individuals exposed to drugs, or whether these changes occur specifically in individuals developing addiction. Pioneering work from the Piazza laboratory addressed this question by comparing synaptic transmission in the NAc of rats that had been classified as either "addict" or "non-addict" using DSM-IV criteria (Kasanetz et al., 2010). Cocaine self-administering rats were classified as "addicts" if they exhibited difficulty in limiting cocaine intake, increased motivation to seek the cocaine and continued use despite adverse consequences. It was found that after 17 days of cocaine self-administration, both "addict" and "non-addict" rats exhibited suppression of NMDA receptor-dependent LTD in the NAc. After 50 days of cocaine self-administration, NMDA receptor-dependent LTD was restored in "non-addict" rats, but these impairments persisted in the "addict" rats, despite no difference in the amount of cocaine these two groups were exposed to Kasanetz et al. (2010). These experiments provide compelling evidence that the transition to addiction may be associated with a form of "anaplasticity," or an inability to counteract drug-induced impairments in synaptic plasticity.

It is apparent from the evidence reviewed above that exposure to drugs of abuse can induce long-lasting changes in synaptic strength in brain regions and circuits associated with drug reward (Hyman et al., 2006; Kauer and Malenka, 2007; Kalivas and O'Brien, 2008; Luscher and Malenka, 2011). In addition to the VTA and NAc, synaptic adaptations upon exposure to drugs have also been characterized in other components of the mesolimbic system including the PFC, bed nucleus of the stria terminalis and central amygdala (Dumont et al., 2005; Fu et al., 2007; Van Den Oever et al., 2008). However, given the above findings it appears that specific deficits in cortico-accumbal synapses of MSNs are the most relevant to addiction in humans.

TRANSCRIPTIONAL MECHANISMS OF DRUG-INDUCED PLASTICITY

While it is clear that drugs of abuse are able to modify synaptic transmission in the mesocorticolimbic system, for stable alterations in neuronal functioning to be achieved, *de novo* protein synthesis is required (Kandel, 2001). Indeed, repeated drug exposure results in region-specific alterations in gene expression and it has been postulated that these changes may underlie some of the enduring behavioral abnormalities that characterize addiction (McClung and Nestler, 2003; Chao and Nestler, 2004). There are a number of mechanisms by which drugs of abuse are able to regulate gene expression, including activation and suppression of transcription factors, epigenetic mechanisms and induction of non-coding RNAs.

TRANSCRIPTION FACTORS

Transcription factors are proteins that bind to specific DNA sequences to regulate gene transcription by interacting with the RNA polymerase II complex (Mitchell and Tjian, 1989). Transcription factors can be induced or repressed in response to environmental stimuli, resulting in changes in gene expression and ultimately neuronal function. A number of transcription factors have been identified for their potential role in addiction because their expression and activation is regulated in the mesocorticolimbic pathway upon exposure to drugs of abuse. ΔFosB is one such transcription factor that has received particular attention due to its unusual stability. ΔFosB is a truncated splice variant of the FosB gene, and it shares homology with other Fos family members including c-Fos, FosB, Fra1, and Fra2 which all heterodimerise with Jun family proteins (c-Jun, JunB, or JunD) to form activator protein-1 (AP-1) transcription factors (Morgan and Curran, 1995). These other Fos family members are induced rapidly in the striatum in response to acute administration of psychostimulants, however due to their instability this expression is transient and returns to basal levels within hours (Graybiel et al., 1990; Young et al., 1991; Hope et al., 1992). Conversely, ΔFosB accumulates in the striatum following chronic drug administration, and its expression persists for several weeks after the last drug exposure (Hope et al., 1994; Nye et al., 1995; Nye and Nestler, 1996; Pich et al., 1997; Muller and Unterwald, 2005; McDaid et al., 2006). Data from behavioral experiments support a role for ΔFosB in some of the lasting effects imparted by drugs of abuse. Over-expression of ΔFosB

in the striatum results in increased locomotor responses to both acute and chronic cocaine, and increases the reinforcing properties of both cocaine and morphine (Kelz et al., 1999; Colby et al., 2003; Zachariou et al., 2006), whereas inhibition of ΔFosB produces the opposite behavioral effects (Peakman et al., 2003). Due to its ability to increase the incentive motivational properties of drugs of abuse, this transcription factor has been proposed to represent a "molecular switch" that facilitates the transition to addiction (Nestler, 2008).

cAMP response element-binding protein (CREB) is another transcription factor that has been the focus of a considerable amount of research due to its proposed role in drug-induced plasticity (McPherson and Lawrence, 2007). CREB is expressed ubiquitously in the brain, and can be activated by a multitude of intracellular signaling pathways that culminate in its phosphorylation at serine 133 (Mayr and Montminy, 2001). Phosphorylated CREB (pCREB) stimulates the recruitment of CREB-binding protein (CBP) which facilitates the transcription of various downstream genes (Arias et al., 1994). pCREB is rapidly induced in the striatum upon exposure to psychostimulants (Konradi et al., 1994; Kano et al., 1995; Walters and Blendy, 2001; Choe et al., 2002) and this is hypothesized to represent a homeostatic mechanism that counteracts behavioral responses to drugs of abuse (McClung and Nestler, 2003; Dong et al., 2006). Consistent with this, overexpression of CREB in the NAc shell reduces the rewarding properties of cocaine in a conditioned place preference (CPP) paradigm, whereas the opposite is observed upon inhibition of CREB in this region (Carlezon et al., 1998; Pliakas et al., 2001). Similarly, genetic knockdown or inhibition of CREB in the dorsal striatum confers increased sensitivity to the locomotor activating properties of psychostimulants, adding further support to this hypothesis (Fasano et al., 2009; Madsen et al., 2012).

While data from CPP experiments support the idea of CREB acting as a negative modulator of drug reward, at least with respect to cocaine, this may be an oversimplification. A number of studies using various techniques to alter CREB function in the NAc shell have revealed that inhibition of CREB reduces cocaine reinforcement in a self-administration paradigm (Choi et al., 2006; Green et al., 2010; Larson et al., 2011), whereas cocaine reinforcement is enhanced by CREB overexpression in this region (Larson et al., 2011). These divergent findings are probably due to fundamental differences between instrumental and Pavlovian conditioning procedures as well as voluntary *vs.* involuntary drug administration. CPP involves associative learning processes, and is thought to be an indirect measure of the hedonic properties of a drug rather than drug reinforcement *per se* (Bardo and Bevins, 2000). Voluntary drug self-administration can be influenced by a number of emotional factors, and the ability of CREB activity in the NAc to reduce responses to anxiogenic stimuli (Barrot et al., 2002) and attenuate depressive behavior (Pliakas et al., 2001) could influence the propensity to self-administer drug. Interestingly, deletion of CREB from the PFC results in decreased motivation to self-administer cocaine (McPherson et al., 2010), demonstrating that the effect of CREB manipulation upon behavior also varies for different brain regions. This is perhaps not surprising given that the CREB transcriptome differs markedly according to the cell type (Cha-Molstad et al., 2004) and it would therefore be important to identify the changes in gene expression occurring down-stream of CREB that contribute to these phenotypes. Complicating things further is the observation that CREB in the NAc shell is essential for nicotine CPP (Brunzell et al., 2009), suggesting that the mechanisms underlying conditioned nicotine reward differ from those underlying cocaine and morphine, which are both enhanced by CREB inhibition in the NAc shell (Carlezon et al., 1998; Pliakas et al., 2001; Barrot et al., 2002).

EPIGENETIC MECHANISMS

Epigenetics has a number of definitions, but in neuroscience it is commonly defined as changes in gene expression that occur through modulation of chromatin which are not brought about by changes in the underlying DNA sequence (McQuown and Wood, 2010). Chromatin describes the state of DNA when it is packaged within the cell. The basic repeating unit of chromatin is the nucleosome, which consists of 147 base pairs of DNA wrapped around an octamer composed of pairs of the four core histones (H2A, H2B, H3, and H4) (Luger et al., 1997). The amino terminal tails of these core histones can undergo a number of post-translational modifications including acetylation, methylation, phosphorylation, ubiquitination, and sumoylation (Berger, 2007). The addition and removal of these functional groups from histone tails is carried out by a large number of histone modifying enzymes, including acetyltransferases, deacetylases, methyltransferases, demethylases, and kinases (Kouzarides, 2007). These histone modifications serve to signal the recruitment of transcription factors and other proteins involved in transcriptional regulation, and alter chromatin conformation to make DNA more or less accessible to the transcriptional machinery (Strahl and Allis, 2000; Kouzarides, 2007; Taverna et al., 2007). Epigenetic mechanisms therefore represent an important means by which environmental stimuli can regulate gene expression and ultimately behavior.

Recently, chromatin modification has been recognized as an important mechanism underlying drug-induced changes in plasticity and behavior (Renthal and Nestler, 2008; Bredy et al., 2010; McQuown and Wood, 2010; Maze and Nestler, 2011; Robison and Nestler, 2011). The first evidence for this came from experiments by Kumar and colleagues who used chromatin immunoprecipitation (ChIP) assays to demonstrate that cocaine induces histone modifications at specific gene promoters in the striatum (Kumar et al., 2005). Specifically, acute administration of cocaine resulted in H4 hyperacetylation of the *cFos* promoter, whereas chronic administration resulted in H3 hyperacetylation of the *BDNF* and *Cdk5* promoters. Histone acetylation involves the enzymatic transfer of an acetyl group to a histone's basic N-terminal tail, which neutralises the electrostatic interaction between the histone and the negatively charged DNA, making it more accessible to the transcriptional apparatus (Loidl, 1994). This is consistent with the ability of cocaine to increase the expression of Fos family transcription factors acutely (Graybiel et al., 1990; Young et al., 1991), whereas BDNF and Cdk5 are induced

only upon chronic exposure (Bibb et al., 2001; Grimm et al., 2003).

A histone hyperacetylated state can also be achieved experimentally by administration of histone deacetylase (HDAC) inhibitors, and these drugs have been used to examine the effects of global increases in histone acetylation upon behavioral responses to drugs of abuse. Systemic administration of HDAC inhibitors synergistically increases the hyperacetylation observed in response to cocaine within the striatum (Kumar et al., 2005), and this potentiates cocaine-induced locomotion and cocaine reward (Kumar et al., 2005; Sun et al., 2008; Sanchis-Segura et al., 2009). HDAC inhibition can also increase locomotor sensitization to ethanol and morphine, and facilitate morphine CPP (Sanchis-Segura et al., 2009), Nevertheless, HDAC inhibitors have also been found to prevent the development of sensitization to a single morphine exposure (Jing et al., 2011), and reduce the motivation to self-administer cocaine (Romieu et al., 2008). These contrasting findings may reflect differences in administration protocols, and importantly they demonstrate that HDAC inhibitors do not indiscriminately potentiate behavioral responses to drugs in all conditions.

Due to their permissive effect upon gene transcription, HDAC inhibitors may also act to facilitate certain types of learning (Bredy et al., 2007; Lattal et al., 2007). It has recently been demonstrated that administration of a HDAC inhibitor following re-exposure to a previously cocaine-paired environment can facilitate extinction of cocaine-induced CPP, and this is probably related to increased histone H3 acetylation in the NAc (Malvaez et al., 2010). Infusion of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) directly into the NAc during the conditioning phase of CPP increases conditioned cocaine reward (Renthal et al., 2007), indicating that HDAC inhibition in this region can facilitate both reward-related learning and extinction learning, depending upon the context in which the drug is administered. Further experiments have revealed a role for HDAC5, and endogenous HDAC expressed highly in the NAc in modulation of cocaine reward. Cocaine administration increases HDAC5 function by regulating its dephosphorylation and subsequent nuclear import, and dephosphorylation of HDAC5 in the NAc impairs the development of a cocaine CPP (Taniguchi et al., 2012). Similarly, over-expression of HDAC5 in the NAc during the conditioning phase of CPP attenuates cocaine reward, and this effect is reversed upon expression of a mutant form of HDAC5 in the NAc (Renthal et al., 2007). It is possible that HDAC5 is exerting these effects by inhibiting drug-induced gene transcription that normally increases the rewarding properties of cocaine.

Genome-wide analysis of chromatin modifications that occur in the NAc as a result of cocaine exposure has revealed a multitude of chromatin modifications at the promoter regions of genes down-stream of both CREB and ΔFosB (Renthal et al., 2009). This analysis also revealed up-regulation of two sirtuins, SIRT1 and SIRT2, which are proteins that possess HDAC activity and can also deacetylate other cellular proteins (Denu, 2005). Induction of SIRT1 and SIRT2 is associated with increased H3 acetylation and increased binding of ΔFosB at their gene promoters, suggesting that they are down-stream targets of ΔFosB (Renthal et al., 2009). The up-regulation of SIRT1 and SIRT2 is thought to have behavioral relevance; sirtuins decrease the excitability of NAc MSNs *in vitro*, and pharmacological inhibition of sirtuins decreases cocaine reward, whereas their activation increases rewarding responses to cocaine (Renthal et al., 2009).

In addition to the functional role for HDACs, genetic studies have also revealed a role for histone acetyltransferases (HATs) in mediating some of the behavioral responses to drugs of abuse. Arguably the most important mechanism by which CBP is able to enhance gene transcription is via its intrinsic HAT activity (Bannister and Kouzarides, 1996), and recent findings implicate the HAT activity of CBP in some of the epigenetic changes that result from drug exposure. In response to acute cocaine, CBP is recruited to the *FosB* promoter where it acetylates histone H4 and increases expression of FosB (Levine et al., 2005). In mice haploinsufficient for CBP, less CBP is recruited to the promoter resulting in decreased histone acetylation and FosB expression. This also corresponds to less accumulation of ΔFosB in the striatum, and not surprisingly these mice exhibit decreased sensitization in response to a cocaine challenge (Levine et al., 2005). Recently, using the cre-lox recombination system Malvaez and colleagues investigated the role of CBP activity located specifically in the NAc upon cocaine-induced gene transcription and behavior (Malvaez et al., 2011). It was reported that targeted deletion of CBP in the NAc resulted in reduced histone acetylation and c-Fos expression, and impaired locomotor activation in response to both acute and chronic cocaine (Malvaez et al., 2011). Conditioned cocaine reward was also inhibited in these mice, providing the first evidence that CBP activity in the NAc is important for the formation of drug-associated memories (Malvaez et al., 2011).

Recently, experiments from the Kandel lab have revealed that epigenetic mechanisms may underlie nicotine's hypothesized ability to act as a "gateway drug". Mice chronically pretreated with nicotine prior to cocaine exposure exhibited enhanced locomotor sensitization and cocaine reward compared to nicotine naive mice (Levine et al., 2011). Additionally, nicotine pretreatment resulted in enhanced cocaine-induced depression of LTP in excitatory synapses in the NAc core, an effect that was not seen with nicotine alone. Analysis of histone modifications induced by 7-day nicotine exposure revealed increased H3 and H4 acetylation at the *FosB* promoter in the striatum, an effect that was not as pronounced in response to 7-day cocaine administration. HDAC activity was reduced in the striatum of nicotine treated mice, but unchanged in mice treated with cocaine. Remarkably, infusion of a HDAC inhibitor directly into the NAc was able to mimic the effects of nicotine pretreatment in potentiating cocaine's effects. None of these changes were observed when mice were treated with cocaine prior to nicotine, confirming the temporal specificity of these effects. This elegant set of experiments has provided a possible epigenetic explanation as to why cigarette smoking almost always precedes cocaine use in the human population (Kandel, 1975; Kandel et al., 1992).

In addition to histone acetylation, histone methylation has also recently been recognized as a behaviorally relevant chromatin modification induced by drugs of abuse (Laplant et al., 2010; Maze et al., 2010, 2011). Histone methylation involves the enzymatic addition of one, two, or three methyl groups to lysine or arginine residues at the N-terminal of histone tails, and is associated with either transcriptional activation or repression, depending upon the nature of the modification (Rice and Allis, 2001). The first studies to examine histone methylation induced by cocaine led to the identification of two histone methyltransferases, G9a and G9a-like protein (GLP), that were persistently down-regulated in the NAc 24 h following both non-contingent cocaine exposure and cocaine self-administration (Renthal et al., 2009; Maze et al., 2010). This down-regulation was linked to similar decreases in histone H3 lysine 9 (H3K9) and 27 (H3K27) methylation. Subsequently, G9a overexpression in the NAc was demonstrated to reduce cocaine-induced expression of selected genes, decrease cocaine reward as measured by CPP, and inhibit the increases in dendritic spine density normally observed in response to repeated cocaine (Maze et al., 2010). The opposite occurred when G9a expression in the NAc was inhibited, resulting in increased dendritic spine density and enhanced cocaine reward. There is evidence that these cocaine-induced changes in G9a expression and subsequent decreases in H3K9 and H3K27 are regulated by ΔFosB (Maze et al., 2010). Collectively, these experiments identified an important role for histone methylation by G9a in some of the long term behavioral and biochemical consequences of repeated exposure to cocaine.

Recently, trimethylation of histone H3 lysine 9 (H3K9me3) which was previously thought to be a relatively stable heterochromatic mark, was shown to be dynamically regulated in the NAc by acute and chronic cocaine exposure (Maze et al., 2011). Repeated cocaine resulted in persistent decreases in repressive H3K9me3 binding which was particularly enriched in non-coding genomic regions (Maze et al., 2011). These initial findings suggest that repeated cocaine exposure may lead to the unsilencing of certain retrotransposable elements in NAc neurons, and it would be of great interest to ascertain the behavioral consequences of these novel epigenetic adaptations.

Given the enduring nature of addiction, recent research has also explored the role of DNA methylation, which is a more stable epigenetic adaptation compared to histone modification. DNA methylation involves the addition of methyl groups to cysteine bases in DNA, and it is generally associated with transcriptional repression (Stolzenberg et al., 2011). Analysis of brains of rats that received passive cocaine injections over 7 days, or that self-administered cocaine over 13 days revealed down-regulation of the DNA methyltransferase DNMT3a in the NAc 24 h after the last cocaine exposure (Laplant et al., 2010). Conversely, following more chronic cocaine exposure (both passive and self-administered for 3 weeks or more) and a 28 day withdrawal period, *dnmt3a* mRNA was found to be significantly enhanced in the NAc (Laplant et al., 2010). Inhibition of DNA methylation/DNMT3a specifically in the NAc was subsequently shown to enhance both CPP and locomotor sensitization to cocaine, whereas the opposite was observed following overexpression of DNMT3a in this region. Moreover, inhibition of DNMT3a in the NAc also prevented cocaine-induced increases in dendritic spine density (Laplant et al., 2010). The behavioral relevance of cocaine-induced alterations in NAc spine density is still not well understood. Manipulations that inhibit drug-induced spine induction have been shown to reduce the rewarding properties of cocaine (Russo et al., 2009; Maze et al., 2010); however, other studies have found that inhibition of spinogenesis potentiates cocaine reward (Pulipparacharuvil et al., 2008; Laplant et al., 2010). As cocaine appears to induce a highly complex regulation of various dendritic spines over the course of exposure and withdrawal (Shen et al., 2009), it has been suggested that these differences may depend upon the type of dendritic spines that are altered (Laplant et al., 2010).

From the experiments described herein, it is clear that drug-induced regulation of the transcriptional potential of cells represents a key mechanism influencing behavioral responses to drugs and reward-related learning. An important next step would be to identify which of these epigenetic changes are most relevant to the human disease state of addiction. Given that mere exposure to drugs is insufficient to produce "addiction" in both humans and animals, the incorporation of models that more closely measure behavioral hallmarks of addiction, such as compulsive drug use and relapse will be of significant value.

MicroRNAs

MicroRNAs represent yet another important means by which drugs of abuse can regulate gene expression. MicroRNAs are small, non-coding RNA transcripts that act to inhibit gene translation at the post-transcriptional level by targeting the 3′-untranslated region (3′UTR) (Bartel, 2004). Recent work by Paul Kenny's group has lead to the identification of transcriptional regulation by microRNAs that occurs specifically in rats with extended access to cocaine self-administration (Hollander et al., 2010; Im et al., 2010). Extended access models precipitate escalating, compulsive patterns of drug-intake which is thought to be reminiscent of the uncontrolled drug use that characterizes human addiction (Ahmed and Koob, 1998; Deroche-Gamonet et al., 2004; Vanderschuren and Everitt, 2004). In rats with a history of extended access to cocaine, the microRNA miR-212 was up-regulated in the dorsal striatum (Hollander et al., 2010), a brain region that becomes progressively engaged with prolonged drug experience (Letchworth et al., 2001; Porrino et al., 2004). Virally-mediated over-expression of miR-212 in the dorsal striatum decreased the motivation to consume cocaine, but only under extended access conditions (Hollander et al., 2010). Inhibition of miR-212 signaling in this region produced the opposite effect, and facilitated compulsive cocaine self-administration. miR-212 is induced in response to CREB signaling (Vo et al., 2005), and exerts its effects by potentiating the activity of CREB (Hollander et al., 2010), revealing a novel feedforward mechanism whereby miR-212 is seemingly able to protect against the development of compulsive cocaine intake.

Expression of the transcription factor MeCP2 is also specifically increased in the dorsal striatum of rats following extended access to cocaine (Im et al., 2010). Disruption of MeCP2 activity in the dorsal striatum prevents the escalation of drug intake normally seen in extended access rats, and results in a progressive decline in responding for cocaine. Unlike CREB and ΔFosB, MeCP2 is a transcriptional repressor, exerting its effects by recruiting HDACs and other transcriptional repressors to silence target genes (Nan et al., 1998). MeCP2 acts to repress expression of miR-212 in the dorsal striatum in an activity dependent manner, and also controls the expression of brain-derived neurotrophic factor (BDNF), a protein with an established role in modulating cocaine-related behaviors (Horger et al., 1999; Graham et al., 2007). miR-212 can also feedback to repress expression of MeCP2, and these two transcriptional regulators are involved in a negative homeostatic balancing act (Im et al., 2010).

These studies highlight the complexity of transcriptional regulation that occurs as a result of drug self-administration, and suggest that voluntary drug intake is controlled by a fine balance of opposing molecular regulators that act to facilitate or inhibit compulsive drug use. It would be of great interest to ascertain whether transcriptional regulation by miR-212/MeCP2 is involved in the mechanism of "recovery" observed in non-addict rats (Kasanetz et al., 2010), and this may bring us closer to understanding factors that underlie both vulnerability and resilience to addiction (Ahmed, 2012).

CONCLUSIONS

Research over the last decade has provided insight into the ability of drugs of abuse to modify synaptic transmission within mesocorticolimbic and corticostriatal circuitry, and we are now beginning to unravel the behavioral significance of some of these changes. More recently, the growing field of epigenetics has shed light upon some of the mechanisms by which drugs of abuse regulate the transcriptional potential of cells, to initiate lasting changes in gene expression. This research has opened up several potential therapeutic avenues. The discovery that N-acetylcysteine is able to restore synaptic deficits induced by self-administration of cocaine, and inhibits reinstatement of drug-seeking offers promise for "rehabilitated" addicts (Moussawi et al., 2011). HDAC inhibitors are gaining attention for their ability to enhance certain types of learning, and the recent discovery that sodium butyrate can facilitate extinction of a cocaine-induced CPP and attenuate reinstatement of drug-seeking is promising (Malvaez et al., 2010). An important next step would be to interrogate the ability of HDAC inhibitors to facilitate extinction of operant self-administration, which more accurately models voluntary drug consumption in humans. Finally, the identification of factors that regulate escalating drug use both on a synaptic level (e.g., persistent impairments in NMDAR-dependent LTD in the NAc) and on a molecular level (e.g., striatal signaling pathways involving miR-212 and MeCP2) are bringing us closer to understanding the mechanisms that underpin the transition to addiction (Hollander et al., 2010; Im et al., 2010; Kasanetz et al., 2010). These studies highlight the importance of examining neuroplastic changes that are brought about by voluntary drug self-administration rather than passive drug exposure. Moving forward it would be important for more research to incorporate these self-administration models that more closely mimic the behavioral pathology seen in human addicts.

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Microglia and drug-induced plasticity in reward-related neuronal circuits

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Drugs of abuse result in complex changes in neurocircuit of reward and stress. Whilst most of these changes affect neurons, the role of additional cell types, such as glia, in the development of tolerance and related neuroplastic changes during drug taking, addiction, and withdrawal is also emerging. For instance, astrocytes, which play an essential role in glutamatergic neurotransmission, as well as in energetic and growth factor support of neurons, are significantly affected by drugs of abuse (Miguel-Hidalgo, 2009). Furthermore, the role of microglia in tailoring neuronal connectivity, transmitter metabolism, and drug-induced neuroinflammation might also be important in physiology of neurons at the addiction-related brain circuitries.

Microglia belong to monocyte/macrophage linage and are resident cells of the innate immune system in the brain. Microglia have at least three, functionally and morphologically distinct forms (Papaleo et al., 2008; Olah et al., 2011). Under normal conditions (1) resting microglia, through their highly mobile filopodia continuously survey the brain parenchyma (Nimmerjahn et al., 2005). Microglia became activated (2) in response to various danger signals posed by neurons and/or astrocytes (Davalos et al., 2005). At this stage, microglia are recruited and have local protective effects via regulated release of cytokines and phagocytosis of cellular debris. At certain point however, microglia gain reactive phenotype (3) characterized by uncontrolled release of inflammatory mediators. These cells furiously attack neurons, became neurotoxic and extend the damage (Banati and Graeber, 1994). It is noteworthy that different insults may converge upon microglial cell population and potentiate each other to worsen the outcome of the response (Zou et al., 2011). However, like macrophages, microglial cells are functionally polarized into different phenotypic activation states, referred as classical (M1) and alternative (M2). Alternatively activated microglia express anti-inflammatory cytokines and involved in tissue protection and repair.

Activated microglia may contribute to addiction-related neuroplastic changes by several ways, such as release of proinflammatory cytokines, synaptic remodeling, neurochemical interaction with excitatory transmission and phagocytosis of newborn neurons and cellular debris.

DRUG-RELATED RECEPTORS ON MICROGLIA

Microglia posses several neurotransmitter and gliotransmitter receptors that might be involved in their activation during drug addiction. Activation of microglia by addictive drugs results in a proinflammatory dominance of the innate immune system, which is then critically synergize on the neurocircuit of reward and dependence (Coller and Hutchinson, 2012). For instance, microglia, similar to other cell of this linage (i.e., macrophages/monocytes), express opioid receptors (Bidlack, 2000; Zou et al., 2011) although their direct role in release of IL-1α and β, TNFα remains to be established. Furthermore, it has recently been demonstrated that morphine binds to an accessory protein (MD-2) of Toll-like receptor 4 (TLR-4) and initiate release of proinflammatory cytokines from microglia and CNS endothelial cells (Wang et al., 2012). By contrast, select phytocannabinoids have anti-inflammatory (Puffenbarger et al., 2000) and neuroprotective (Martin-Moreno et al., 2011) effects that are mediated by cannabinoid receptors (CB1R and/or CB2R) which have been identified on microglia (Cabral and Marciano-Cabral, 2005; Racz et al., 2008).

Acute and chronic exposure to alcohol also results in region specific activation of glial cells (astrocytes and microglia) in dose- and time-dependent manner (Crews et al., 2011). Recent work in TLR-4 deficient mice highlighted the critical role of lipid rafts, TLR-4 and its interaction with MD-2 and CD14 accessory proteins in alcohol-induced neuroinflammation (Alfonso-Loeches et al., 2011; Coller and Hutchinson, 2012).

It is noteworthy that other abused drugs such as cocaine and methamphetamine also provoke proinflammatory immune signaling in the CNS (Lee et al., 2009), suggesting that neuroinflammation is indeed a general and critical component in the development and maintenance of drug abuse. To support this hypothesis it has been demonstrated that agents blocking microglia activation (minocycline, ibudilast) could inhibit drug-induced cytokine, chemokine, and behavioral responses (Hutchinson et al., 2008, 2009; Agrawal et al., 2011; Schwarz et al., 2011). For instance, it has recently been shown that p38 signaling in the microglia in the nucleus accumbens is involved in acquisition and maintenance of morphine-induced conditioned place preference (CPP) that can be suppressed by microglia inhibitors (Zhang et al., 2012).

On the other hand, opiate antagonist-based addiction therapy inhibits innate immune gene expression (Liu et al., 2000). It should be noted, however, that neuroinflammation *per se* does not result in addiction, however may worsen drug effects in addicted individuals (Bruce-Keller et al., 2008).

MICROGLIA AND DRUG-INDUCED MORPHOLOGICAL PLASTICITY

It is well documented that exposure to various addictive drugs produces permanent morphological and physiological changes in dendrites, dendritic arborization, dendritic spines, and synaptic density in brain regions that are implicated in reward, decision making, and inhibitory control of behavior. For instance, stimulants such as cocaine, amphetamine, and nicotine (self-administration or repeated dosing) result in an increase of

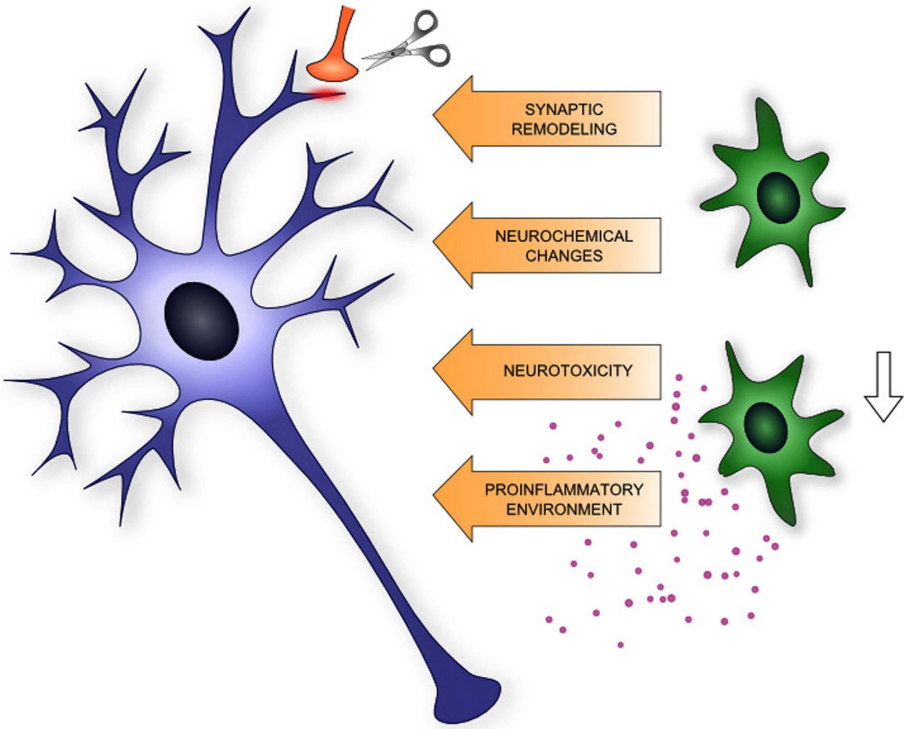

FIGURE 1 | Schematic summary of microglia (shown in green) – neuron (shown in blue) interaction relevant to neuroplasticity in addiction.

spine density on medium spiny neurons of the nucleus accumbens and on the apical dendrites of pyramidal neurons of the prefrontal cortex (Robinson and Kolb, 1999, 2004). On the other hand, depressants, such morphine, significantly reduce spine densities and dendritic branching in these areas (Robinson and Kolb, 1999). Considerable amount of information has been accumulated over the years on the cellular and molecular mechanisms that govern drug experience-induced functional and morphological plasticity (Nestler, 2004; Hyman et al., 2006). With recent emergence of microglia as effectors in neural circuit reorganization, "a new player" should be considered in drug-induced neuronal plasticity (Graeber, 2010). During development, microglia engulf, and phagocytose synapses via fractalkine–fractalkine receptor (CX3CR1) dependent manner, and play an important role in synaptic "pruning" in postnatal brain development (Paolicelli et al., 2011). It has recently been suggested that microglia may actively contribute to the experience-dependent modification or elimination of a specific subset of synapses in the adult healthy brain as well (Graeber, 2010). Indeed, microglial processes make specific contacts with synapses especially with presynaptic elements and the timing of these contacts is activity-dependent (Wake et al., 2009). Recent *in vivo*, two photon imaging and electron microscopy studies have revealed that microglia processes were associated with dendritic spines in experience-dependent manner, supporting the role of microglia in synaptic remodeling (Wake et al., 2009; Tremblay, 2012; Tremblay et al., 2012). Based on the significant remodeling of drug-related brain areas and the role that microglia plays in morphological plasticity during development and in the healthy adult brain, one can hypothesize microglia significantly contribute to morphological and physiological synaptic plasticity in the addicted brain as well.

MICROGLIA AND DRUG-INDUCED NEUROCHEMICAL CHANGES

Evidences have been accumulated over the years to support the involvement of glutamatergic neurotransmission in the mechanisms of drug dependence involving the dopaminergic reward circuit. Microglia contain enzyme machinery [indoleamine 2,3 dioxygenase (IDO)] to produce quinolinic acid (QUIN). QUIN promotes glutamate release through activation of *N*-methyl-D-aspartate (NMDA) receptors. Quinolinic acid also induces oxidative stress, which in combination with glutamate release may contribute to CNS excitotoxicity. For instance in alcoholic patients ethanol may generate significant levels of, quinolinic acid, possibly even toxic levels in localized brain areas (Morgan, 1991).

MICROGLIA AND DRUG-INDUCED NEURODEGENERATION

Microglia play a pivotal role in maintaining the balance between neurogenesis and neuronal cell death in the brain via phagocytosis of apoptotic cells and necrotic debris. Neuronal stem cells and progenitors in the subgranular zone of the hippocampus give rise to newborn granular cells into the hippocampal circuit to participate learning, memory, fear, and mood regulation that are also important aspects of the addictive behavior. Under quiescent conditions microglia is ramified and seems to be neuroprotective, supporting neurogenesis through their interaction with T cells and by expressing MHCII and the neurotrophic factor insulin growth factor (IGF)-1. At this stage, microglia is very efficiently engulf apoptotic cells. However, addictive drugs decrease neurogenesis in the dentate gyrus of the hippocampus and

in the prefrontal cortex (Eisch and Harburg, 2006) and activated microglia might be involved in this process as well. It has been hypothesized that new neurons may block memories associated with the contextual reinstatement of drug seeking or enhance extinction learning. Thus, the reduction in neurogenesis (i.e., a reduction in neuronal turnover) that is observed after self-administration of various drugs of abuse may result in a more robust and long-lasting memory of drug taking and seeking or decrease extinction learning (for review: Mandyam and Koob, 2012). In response to drug-induced microglia activation and proinflammatory environment the phagocytic potential of the microglia is further enhanced, leading to neurodegeneration.

In summary, microglia are emerging contributors of drug-induced morphological and physiological plasticity by promoting neuroinflammation through release of pro-inflammatory cytokines, by their active role in synaptic remodeling, involvement in excitotoxic neurochemical changes, and by phagocytic activity of newborn progenitors (**Figure 1**). Based on these facts microglia might be a potential target for the therapy of drug addiction.

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Neuropeptides as mediators of the early-life impact on the brain; implications for alcohol use disorders

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The brain is constantly exposed to external and internal input and to function in an ever-changing environment we are dependent on processes that enable the brain to adapt to new stimuli. Exposure to postnatal environmental stimuli can interfere with vital adaption processes and cause long-term changes in physiological function and behavior. Early-life alterations in brain function may result in impaired ability to adapt to new situations, in altered sensitivity to challenges later in life and thereby mediate risk or protection for psychopathology such as alcohol use disorders (AUD). In clinical research the studies of mechanisms, mediators, and causal relation between early environmental factors and vulnerability to AUD are restricted and attempts are made to find valid animal models for studies of the early-life influence on the brain. This review focuses on rodent models and the effects of adverse and naturalistic conditions on peptide networks within the brain and pituitary gland. Importantly, the consequences of alcohol addiction are not discussed but rather neurobiological alterations that can cause risk consumption and vulnerability to addiction. The article reviews earlier results and includes new data and multivariate data analysis with emphasis on endogenous opioid peptides but also oxytocin and vasopressin. These peptides are vital for developmental processes and it is hypothesized that early-life changes in peptide networks may interfere with neuronal processes and thereby contribute the individual vulnerability for AUD. The summarized results indicate a link between early-life rearing conditions, opioids, and ethanol consumption and that the ethanol-induced effects and the treatment with opioid antagonists later in life are dependent on early-life experiences. Endogenous opioids are therefore of interest to further study in the early-life impact on individual differences in vulnerability to AUD and treatment outcome.

Keywords: maternal separation, early-life environment, dynorphin, enkephalin, endogenous opioids, oxytocin, vasopressin, addiction

INTRODUCTION

The brain is constantly exposed to external and internal input and to be able to function in an ever-changing environment we are dependent on processes that enable the brain to adapt to new stimuli. The ability to adapt to new conditions is particularly important during development. The developmental processes throughout the prenatal period and the final postnatal reorganization and maturation include a variety of adaptation processes that shape brain function (Crews et al., 2007; Crews, 2008; Turrigiano, 2008). During these developmental time windows the brain is highly sensitive to environmental input. Environmental factors interact with the genome through epigenetic or transcriptional mechanisms and result in long-term alterations in basal function and neuroplasticity (de Kloet et al., 2005; Holmes et al., 2005; Crews, 2008). Such early-life alterations may be favorable for the individual but may also result in impaired ability to adapt to new situations and in altered sensitivity to challenges later in life and thereby contribute to the individual vulnerability for later disease (Nemeroff, 2004; Gluckman et al., 2007; McCrory et al., 2011).

Environmental influences, particularly during childhood and adolescence, have profound impact on the liability to develop alcohol use disorders (AUD) (De Bellis, 2002; Langeland et al., 2004). However, the mechanisms and mediators of the early-life impact on development of AUD are poorly understood. The transition from habitual drug taking into the addictive state may be affected but the mechanisms underlying the individual differences in these transition processes are not clear (Everitt and Robbins, 2005; Koob and Volkow, 2010). Studies of causal relationships between early environmental factors and later vulnerability or resilience to addiction are restricted in clinical research. How can we, for example, distinguish and establish relations between the impact of innate factors, early-life adversity, and early-life drug consumption in an individual that have been diagnosed AUD? To that end we need valid animal models where we can simulate early-life adverse and naturalistic conditions, respectively, and study the consequences later in life. The present article focuses on rodent models and environmentally induced changes in peptide networks within the brain and pituitary gland after exposure to maternal separation (MS) for short or prolonged

periods during the postnatal period. Several lines of evidence from rodent MS studies support the notion that the early-life rearing conditions have long-term consequences for ethanol consumption (Weinberg, 1987; Hilakivi-Clarke et al., 1991; Huot et al., 2001; Ploj et al., 2003a; Jaworski et al., 2005; Gustafsson and Nylander, 2006). The results are not conclusive and it is evident that the effects on ethanol consumption are highly dependent on the experimental paradigm (Jaworski et al., 2005; Roman and Nylander, 2005; Moffett et al., 2007). However, most studies report higher voluntary consumption after prolonged separations as compared to the low ethanol consumption seen after short periods of MS (see reviews by Roman and Nylander, 2005; Moffett et al., 2007). The results summarized herein show that environmentally induced changes in opioid networks can contribute to differences in ethanol consumption patterns later in life and that the variability in outcome may relate to the fact that different MS paradigms cause distinct effects on basal and ethanol-induced effects on opioid peptides.

Importantly, we do not focus on the addicted brain and the alterations discussed are not consequences of compulsive ethanol use. The emphasis is on the neurobiological alterations induced by early-life conditions as a cause for risk consumption and vulnerability to AUD. The article provides a review of earlier results and also includes new data from experimental studies that investigated the impact of early-life conditions on neuropeptides. Emphasis is on endogenous opioid peptides and to some extent oxytocin and vasopressin. These peptides are known to be vital for normal social, emotional, and neurobiological development (Nelson and Panksepp, 1998) and this review summarizes findings showing that early-life experiences induce pronounced changes in basal levels of these peptides, especially the opioid peptide Met-enkephalinArg^{6}Phe^7 (MEAP) and oxytocin. Furthermore, it is shown that the ethanol-induced effects on opioids and the efficacy of opioid antagonists to reduce voluntary ethanol consumption are dependent on early-life history. The compiled results support the hypothesis that early-life changes in basal opioid peptide functioning may contribute to the individual vulnerability or resilience to develop AUD.

TARGETS WITHIN NEUROPEPTIDE CIRCUITS

Environmental factors interact with innate factors and result in a specific response depending on the individual genotype. So far, the molecular (epigenetic and transcriptional) mechanisms underlying these interactions are unclear and, likewise, the mediators (targeted proteins) of the environmental influences are not fully known. There are a number of different proteins associated with peptide transmission and they are all putative targets for environmental influence. Peptides are synthesized in the neuron from precursor proteins and the peptides act on specific receptor proteins. A number of enzymes participate in peptide turnover; processing enzymes cleave the precursor proteins into active peptides, converting enzymes participate in the conversion of one bioactive peptide into another peptide that may have other effects through actions on another receptor, and inactivating enzymes metabolize peptides into inactive peptide fragments (Hallberg and Nyberg, 2003; Hallberg et al., 2005; Nyberg and Hallberg, 2007). Environmental influences may cause altered activity in any of the genes encoding for the precursor proteins, the receptors, and/or the enzymes. Changes in any of these proteins may contribute to altered activity in neuronal circuits that utilize peptides as transmitters and in networks that are modulated by peptides. The present review focuses on the effects on the products of the genes with emphasis on peptides, particularly the opioid peptides.

ENDOGENOUS OPIOIDS

After the first reports of endogenous opioid receptors (Pert and Snyder, 1973; Simon et al., 1973; Terenius, 1973) three distinct receptors have been described, the mu-opioid peptide receptor (MOPR), the delta-opioid peptide receptor (DOPR), and the kappa-opioid peptide receptor (KOPR) (Kieffer and Evans, 2009). Opioid receptors are widely distributed throughout the neuroaxis, and although the localization is similar to some extent, the different receptor types display specific anatomical distributions (Mansour et al., 1988; Akil et al., 1998; Kieffer and Evans, 2009). The opioid peptide family includes endorphins, enkephalins (ENKs), and dynorphins (DYNs) and they bind with different selectivity to MOPR, DOPR, and KOPR, respectively (Akil et al., 1998; Terenius, 2000). Endogenous opioids originate from the precursor proteins proopiomelanocortin (POMC), proENK, and proDYN (Nakanishi et al., 1979; Kakidani et al., 1982; Noda et al., 1982). Each precursor protein is the product of a distinct gene and is enzymatically cleaved to several peptide products. ProENK is the precursor of multiple ENKs such as Leu-ENK, Met-ENK, and MEAP. ProDYN generates dynorphin A (DYNA), dynorphin B (DYNB), and neoendorphin. The main sites for POMC biosynthesis are the pituitary gland that releases opioids into the circulation, and the hypothalamus in neurons that project to a number of other brain areas (Smith, 2006). Neurons expressing proENK and proDYN, respectively, form pathways that are found on almost all levels in the central nervous system (CNS) and in the pituitary gland (McLaughlin, 2006; Spampinato, 2006). Co-expression of neuropeptides is common; DYNs are for example co-expressed with vasopressin in the neurosecretory hypothalamic neurons that project to the neurointermediate lobe of the pituitary gland (Summy-Long et al., 1984). The opioids are involved in basal functions such as motivation, reproductive behavior, food and fluid intake, but also in analgesia, stress reactivity, learning and memory, reward and reinforcement, motor function, and endocrine regulation (Van Ree et al., 1999; Kieffer and Gaveriaux-Ruff, 2002; Przewlocki, 2002; Trigo et al., 2010; Bodnar, 2011). The opioid peptides also serve as neuromodulators and the opioid regulation of dopaminergic pathways (Christensson-Nylander et al., 1986; Spanagel et al., 1992; Steiner and Gerfen, 1998) is of special interest with respect to drug reward, reinforcement, and addiction. The opioid receptors mediate different, often opposite, effects on dopamine transmission. Activation of the KOPR is for example associated with dysphoria whereas the MOPR and DOPR mediate euphoric effects (Akil et al., 1998) and KOPR activation results in reduction in extracellular levels of dopamine in the nucleus accumbens whereas MOPR agonists increase dopamine, presumably through actions in the VTA (Shippenberg et al., 1992; Spanagel et al., 1992; Herz, 1998; Zapata and Shippenberg, 2006). DYNs also regulate the striatonigral dopamine pathway and here DYNB has been suggested

to exert negative feedback control, a specific effect that is distinct from that of the ENKs (Christensson-Nylander et al., 1986; Herrera-Marschitz et al., 1986). The enzymatic conversion of DYNs to Leu-enkephalinArg^6 (DYN1-6) and Leu-ENK is therefore of special interest since it results in loss of KOPR-mediated effects in favor of DOPR-mediated effects (Hallberg et al., 2005). Consequently, any change in this enzymatic step will also change the physiological output from proDYN circuits.

The endogenous opioid peptides are present in the CNS well before birth and are among the first neurochemical markers to be detectable in the developing brain (Tohyama, 1992). During the first few weeks of neonatal life a significant developmental reorganization of the opioid systems occurs accompanied by regional divergence within the brain (McDowell and Kitchen, 1987; Morita, 1992; Leslie and Loughlin, 1993). The MOPR and KOPR are the first binding sites to appear within the rodent CNS at the embryonic stage. During the first few weeks of life the number of MOPR and KOPR increases substantially before declining to adult levels whereas the DOPR are expressed later with the highest expression during the second postnatal week (Spain et al., 1985; Petrillo et al., 1987). The presence of opioids when neurogenesis, neuronal migration, process outgrowth, and synaptogenesis occur suggests a role for opioids in neuronal developmental processes.

Opioids modulate social behavior, especially social interactions early in life, and several lines of evidence support a functional role of opioids in parental behavior and social bonding processes (Panksepp et al., 1980, 1994; Nelson and Panksepp, 1998). The finding of deficient attachment behavior in mice lacking the MOPR further strengthened the concept of opioid involvement in infant attachment behavior (Moles et al., 2004). Mouse pups lacking the MOPR gene had altered attachment behavior toward their mothers. These knockout mouse pups emitted less ultrasonic vocalizations when removed from the mother and, in addition, the preference toward their mothers' cues was abolished (Moles et al., 2004).

OXYTOCIN AND VASOPRESSIN

The oxytocin and vasopressin receptors are detected in the hypothalamus and the amygdala but also in other brain areas (Barberis and Tribollet, 1996; Gimpl and Fahrenholz, 2001). So far, one oxytocin receptor and three vasopressin receptors have been described (Jard et al., 1987; Kimura et al., 1992; Lolait et al., 1995; Morris, 2006). The genes encoding oxytocin and vasopressin are present on the same chromosome and the precursor proteins are predominantly expressed in neurons within the paraventricular and supraoptic nuclei of the hypothalamus with projections to a number of target areas (Buijs, 1983; Gimpl and Fahrenholz, 2001; Landgraf and Neumann, 2004). However, expression also occurs in the amygdala and other brain areas (De Vries and Buijs, 1983; Planas et al., 1995; Chodobski et al., 1998; Hallbeck et al., 1999; Morris, 2006). The magnocellular neurons project to the neurointermediate lobe of the pituitary gland and release peptides into the circulation and the parvocellular neurons project to the median eminence and release peptides into the portal circulation (Buijs, 1992; Gimpl and Fahrenholz, 2001). Neuropeptides may also diffuse over long distances in the extracellular fluid (Landgraf and Neumann, 2004) and may interact with distant receptors.

Oxytocin and vasopressin are involved in a number of physiological and behavioral functions through peripheral and central actions (Gimpl and Fahrenholz, 2001). Central actions include involvement in memory processes (Dantzer et al., 1987), anxiety (McCarthy et al., 1996; Bale et al., 2001; Ring et al., 2006), and regulation of emotional and social behavior in males and females (Young and Wang, 2004; Heinrichs and Domes, 2008; Neumann, 2008; Skuse and Gallagher, 2009). A number of studies have investigated the role of oxytocin in prairie voles that are highly affiliative, forms enduring social bonds between mates and displays bi-parental behavior, which is contrasting to rats and mice but more similar to humans. These studies have identified a neural circuitry model of social bonding including oxytocin, vasopressin, and dopamine as important target systems (Ahern and Young, 2009; McGraw and Young, 2010). Oxytocin and vasopressin also regulate stress responses (Neumann, 2002; Kramer et al., 2003; Engelmann et al., 2004; Landgraf and Neumann, 2004) and are affected by stressful stimuli (Hashiguchi et al., 1997; Ebner et al., 2005; Aguilera et al., 2008).

Oxytocin and vasopressin networks continue to develop during the early postnatal and adolescent period (Shapiro and Insel, 1989; Buijs, 1992; Lipari et al., 2001). These peptides have important roles early in life, for example they are vital for the establishment of early social behavior and several reports describe the role for oxytocin and vasopressin in maternal behavior, mother-pup interactions, and social bonding (Insel and Shapiro, 1992a,b; Nelson and Panksepp, 1998; Insel, 2003; Carter et al., 2008; Ahern and Young, 2009; McGraw and Young, 2010). Early-life experiences may interfere with these developmental processes and result in long-term neurobiological and behavioral consequences. Altered levels have for example been detected in children that have experienced early adversity (Fries et al., 2005; Heim et al., 2009).

ANIMAL MODELS FOR STUDIES OF EARLY-LIFE IMPACT ON BIOLOGY AND BEHAVIOR

The environmental conditions during the first postnatal weeks are critical for a normal development in the rodent. The first two postnatal weeks, starting within a few days after birth, are referred to as the stress hyporesponsive period since the adrenal responses to stress are blunted with little or no corticosterone release and the glucocorticoid receptors undergo critical developmental stages (Sapolsky and Meaney, 1986). The newborn rat is dependent on the mother for survival and normal development and the level of maternal licking and grooming behavior toward the pups during the first postnatal week has profound impact on their adult hypothalamus-pituitary-adrenal (HPA) axis response to stress (Levine, 2002; Weaver et al., 2004; Holmes et al., 2005). Other developmental processes are also affected; 360 min daily MS alter for example the ultrasonic vocalization trajectory (Ploj et al., 2003b) and delay eye-opening (Ploj et al., 2002). The interaction between the CNS and the endocrine system is manifested during development and may serve to "program" behavioral and neuroendocrine functions (de Kloet et al., 2005). Disturbance of the social interactions during these first postnatal weeks may therefore interfere with critical developmental processes and result in

persistent changes in brain function and behavior and thereby determining the adult phenotype.

EARLY HANDLING/NEONATAL HANDLING AND MATERNAL SEPARATION

Early studies showed positive effects in adult rats that had been handled daily for 10 min during the first three weeks of life (Weininger, 1954) and that daily separations of mother and pups for only three minutes, "early handling," reduced physiological responses to stress (Levine, 1957; Levine and Lewis, 1959). Several later studies confirmed that rats subjected to brief handling displayed a hyporesponsive stress response as compared to non-handled rats (Ader and Grota, 1969; Anisman et al., 1998; Meaney, 2001; Pryce and Feldon, 2003). These studies initiated extensive research on the effects of early-life events in rodents and they were also the starting point for the use of MS as an experimental model in studies on the biological and behavioral consequences of early environmental factors.

In MS models, the separation of the mother and the pups is commonly performed during the first two to three postnatal weeks. Both shorter and prolonged separations are denominated MS in the literature, for example MS15 for a short 15 min separation and MS180, MS240, or MS360 for 180–360 min separations (**Figure 1**). Maternal deprivation usually refers to 24 h of maternal absence. The experimental design in MS paradigms aims to create early adversity by repeatedly separating the rat pups from the dam for longer periods of time, commonly 180 min or longer. Prolonged separations disrupt the early social mother-pup interactions that are vital for normal neuronal and behavioral development and are regarded as a risk environment associated with early-life stress and negative consequences later in life (Ladd et al., 2000; Levine, 2002; Pryce and Feldon, 2003; Holmes et al., 2005).

Although a number of MS protocols are in use and several authors have described the problem with the variability in outcome (Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Jaworski et al., 2005; Roman and Nylander, 2005) the accumulated knowledge from these studies has improved our general understanding of the effects of various early-life conditions on the brain and behavior. The use of different control groups when assessing the consequences of prolonged MSs is one reason for variable results (see e.g., Lehmann and Feldon, 2000; Levine, 2002; Pryce and Feldon, 2003; Roman and Nylander, 2005; Macri and Würbel, 2006). Common control groups and MS groups are depicted in **Figure 1**. Non-handling refers to a condition with no experimenter contact during the first postnatal weeks and no cleaning/changes of the cages. Animal facility rearing (AFR) refers to conventional animal facility housing with experimenter contact only when the cages are changed. Although conventional housing is similar between laboratories there are still several possible confounding factors due to different laboratory environments. Furthermore, often it is not reported whether there are one or several experimenters participating in the separation procedures and not how the contacts are made. Non-handling and AFR are clearly not similar to a normal rodent rearing environment and the non-handling paradigm has even been suggested to be stressful (Pryce and Feldon, 2003; Macri and Würbel, 2006). The mother and pups are constantly together and the animals are

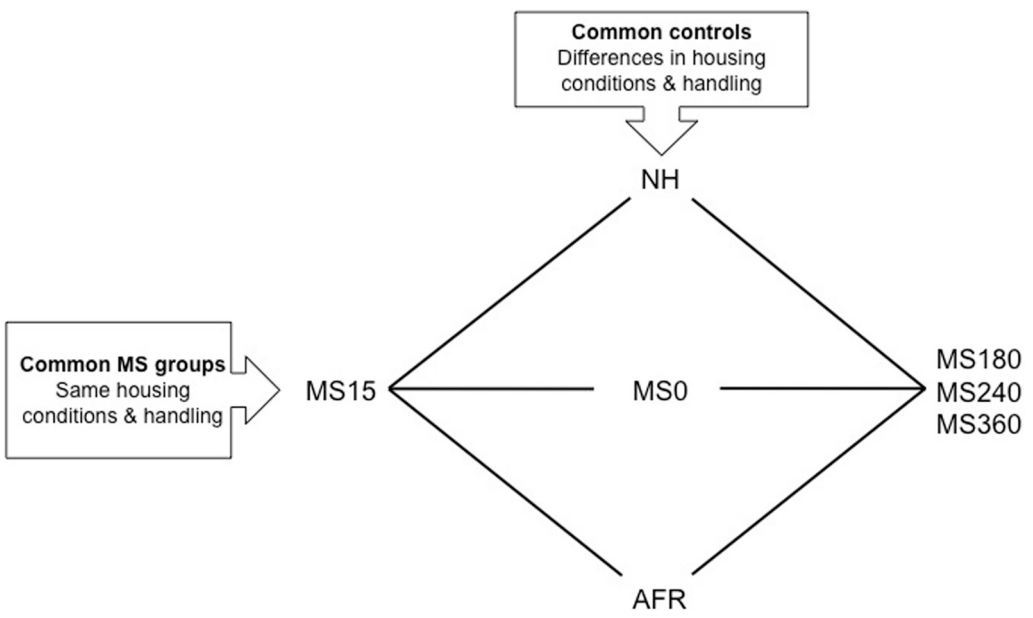

FIGURE 1 | A schematic outline of different experimental groups in rodent maternal separation (MS) paradigms. Rats in the horizontal groups are subjected to the same handling by the experimenter and the same housing conditions. With the use of these groups the effect of being separated from the dam different length of time can be assessed. The rat pups can be exposed to either absence of the dam (litter-wise MS) or absence of the dam and the littermates (individual MS). The vertical groups represent examples of control groups commonly used in MS studies. These groups differ in a number of aspects and care must be taken in choosing the proper control depending on the question asked. NH, non-handling; AFR, animal facility rearing; MS0, brief handling of the pup; MS15, 180, 240, 360, maternal separation for 15, 180, 240, or 360 min.

subjected to few environmental stimuli compared to wildlife conditions. In addition, the handling during AFR and non-handling differs from the MS condition and it is not possible to distinguish between effects induced by handling and separation (**Figure 1**).

The use of briefly handled rats as control to prolonged separation reduces the problem with different handling. In the literature, brief handling procedures include MS0 that usually refers to separation less than a minute, or handling for 1–5 min (Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Jaworski et al., 2005; Roman and Nylander, 2005). Based on the similarity to wildlife rearing conditions where the lactating dam leaves the nest regularly, often around 10 min and not longer than one hour depending on the age of the offspring (Grota and Ader, 1969), repeated shorter separations (3–15 min) are commonly used to simulate a beneficial environment related to positive behavioral consequences in studies of protective factors. Comparisons between short and prolonged separations also enable analysis of the effects of the duration of maternal absence without confounding effects of other experimental conditions, such as handling by the experimenter and general housing conditions (**Figure 1**).

One example of different outcome in MS studies is the effects on the HPA axis. HPA axis function is clearly affected by early handling and separation procedures (Anisman et al., 1998; Lehmann and Feldon, 2000; Pryce et al., 2002, 2005; Macri and Würbel, 2006) but the effects are not conclusive and highly dependent on the control group (see Pryce and Feldon, 2003; Macri and Würbel, 2006). Early handling results in a hyporesponsive HPA axis response compared to non-handling whereas prolonged separations results in a hyperresponsive HPA axis but only in comparison to early handling and not compared to non-handling (Ladd et al., 2000; Lehmann and Feldon, 2000; Meaney, 2001; Pryce and Feldon, 2003; Nemeroff, 2004). More recent studies have shown a blunted corticosterone response after prolonged separations compared to animals subjected to AFR or short separations (Greisen et al., 2005; Kim et al., 2005; Roman et al., 2006).

Finally, the outcome of MS is also dependent on experimental parameters such as the origin of animals. Recent studies have described profound differences in behavior (Palm et al., 2011) and in basal and ethanol-induced levels of opioids (Palm et al., 2012) in Wistar rats from different suppliers. Temperature, size, and sex composition of the litters, if separations are performed daily or at randomly chosen days, etc., are other examples of parameters that affect the outcome (Lehmann and Feldon, 2000; Pryce and Feldon, 2003; Roman and Nylander, 2005). Prolonged separations may alter maternal care and several authors have discussed whether the effects seen after MS are due to the loss of maternal contact, caused by an altered behavior directed toward the pup, or both (Liu et al., 1997; Caldji et al., 1998; Francis et al., 1999; Pryce et al., 2001; Marmendal et al., 2004; Macri and Würbel, 2006; Eklund et al., 2009; Daoura et al., 2010).

THE EFFECTS OF EARLY-LIFE CONDITIONS ON BASAL LEVELS OF NEUROPEPTIDES

Since the effects induced by rearing in the risk environment (repeated prolonged separations) depend on whether AFR, non-handling or short repeated separations are used as controls the following section is divided into two parts, one section ("Maternal Separation vs. Non-handled Rats or AFR Rats") describing the effects of MS in comparison to either non-handled rats or rats subjected to AFR, and the following section ("Short vs. Prolonged Maternal Separation") showing the effects of prolonged MS compared to short MS.

MATERNAL SEPARATION VS. NON-HANDLED RATS OR AFR RATS

Several studies show that MS affects DYNB levels and differences have been described both in comparison with non-handled rats (Ploj et al., 1999) and AFR rats (Ploj et al., 2003b; Gustafsson et al., 2008). Robust effects have been described in the pituitary gland with higher DYNB levels in adult rats after daily individual 15 min MS compared to non-handled rats (Ploj et al., 1999) and after either litter-wise or individual separations compared to AFR (Ploj et al., 2003b; Gustafsson et al., 2008). Previously described MS-induced effects on DYNB in various brain areas are not entirely conclusive (Ploj et al., 1999, 2001, 2003b) and the impact of age and different rearing conditions on the outcome was specifically addressed in a recent comparative study. The basal levels were assessed after either individual or litter-wise MS for either 15 or 360 min in 3- and 10-week-old rats in one experiment with one experimenter, the same animal housing conditions and the same rat strain and supplier (Gustafsson et al., 2008). This study revealed both immediate (**Figure 2**) and long-lasting (**Figure 3**) effects on DYNB and also distinct effects of litter-wise MS (**Figures 2A**,**3A**) and individual (**Figures 2C**,**3C**) MS, respectively, but when compared to AFR the effects were similar after MS15 and MS360. MS was again found to increase DYNB levels in the pituitary gland. In the hypothalamus no differences were detected between the MS and AFR rats. These results contrast the finding of higher DYNB after individual MS15 compared to non-handled rats (Ploj et al., 1999) and in litter-wise MS15 compared to AFR in adult single-housed rats (Ploj et al., 2003b). Interestingly this effect was clearly sex dependent; in female rats MS15 instead resulted in decreased hypothalamic DYNB (Ploj et al., 2001). These results indicate that the effects on DYNB in the hypothalamus are only detectable when an additional stress-component was added to the repeated maternal absence, such as single housing. Compared to AFR, MS induced opposite effects in the nucleus accumbens and dorsal striatum in young rats (**Figure 2**); a pronounced reduction in DYNB levels was seen in the nucleus accumbens both after litter-wise and individual MS whereas in the striatum, individual MS caused markedly higher DYNB levels (Gustafsson et al., 2008). In adult rats (**Figure 3**), differences between MS and AFR were instead found in the substantia nigra after litter-wise MS and in the ventral tegmental area (VTA) after individual MS. The different effects in the ventral and dorsal striatum and in the substantia nigra and VTA where DYNB is known to modulate the mesolimbic and nigrostriatal dopamine pathways (Christensson-Nylander et al., 1986; Herrera-Marschitz et al., 1986) indicate that handling procedures during MS may affect the opioid regulation of dopamine transmission.

The opioid peptide MEAP seems to be highly sensitive to handling and separation procedures during the postnatal period (Ploj et al., 2003b; Gustafsson et al., 2007, 2008). The effects

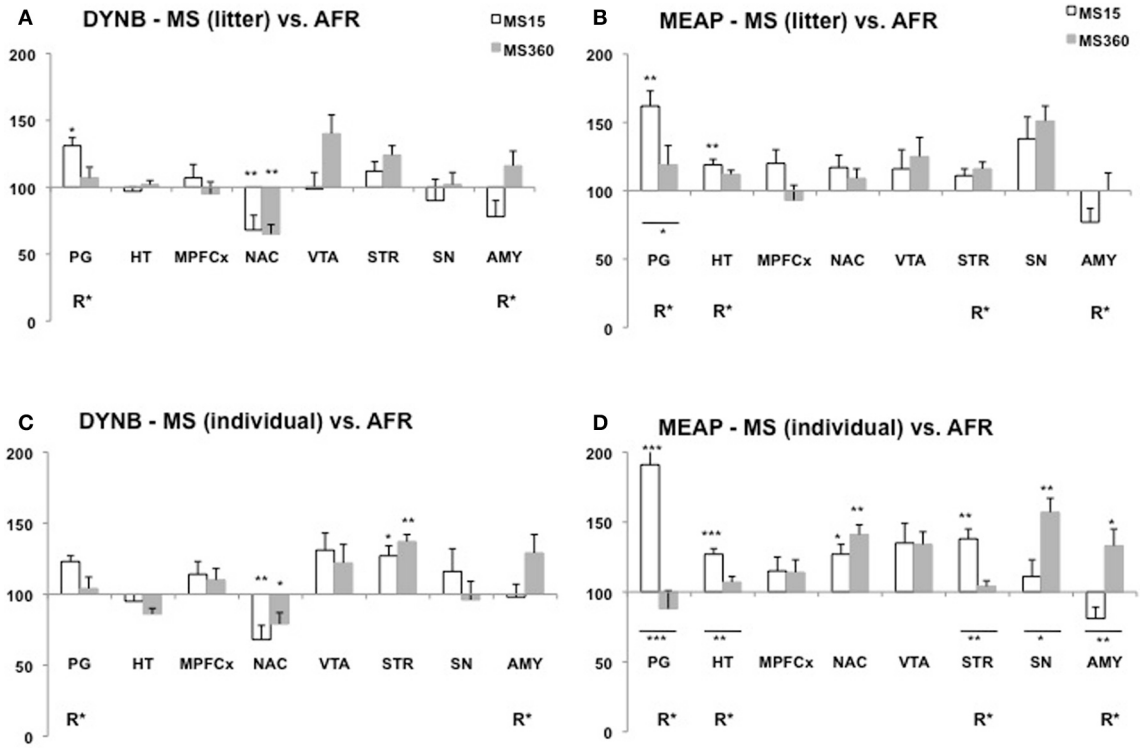

FIGURE 2 | Dynorphin B (DYNB) and Met-enkephalin-Arg^{6}Phe^7 (MEAP) in 3-week-old rats after exposure to different postnatal conditions. Immunoreactive levels of DYNB **(A,C)** and MEAP **(B,D)** in rats subjected to MS15 or MS360 in the litter-wise MS paradigm **(A,B)** and in the individual MS paradigm **(C,D)**. The peptides were analysed at three weeks of age, immediately after the MS period. Values represent mean ± standard error of the mean and are shown as percent of the peptide levels in animals subjected to animal facility rearing (AFR) conditions (data from Gustafsson et al., 2008). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Comparison with peptide levels in the AFR group. *Under a line, comparison between the MS15 and MS360 groups. R*, main effect of rearing environment, i.e., difference between all MS15 rats (litter-wise and individual separation) and all MS360 rats (litter-wise and individual separation). Abbreviations: PG, pituitary gland; HT, hypothalamus; MPFCX, medial prefrontal cortex; NAC, nucleus accumbens; VTA, ventral tegmental area; STR, dorsal striatum; SN, substantia nigra; AMY, amygdala.

are region-specific, dependent on separation conditions and the age when the effects were examined (Gustafsson et al., 2008). In the pituitary gland both litter-wise and individual MS15 resulted in higher MEAP levels as compared to AFR in 3-week old rats (**Figure 2**). In the brain, the individual and litter-wise MS resulted in distinct effects. Individual MS produced changes that were detectable in basal levels immediately after the MS period (**Figure 2D**); higher levels were seen after daily MS15 in the hypothalamus, nucleus accumbens, striatum, and higher levels after daily MS360 in the frontal cortex (not shown in the figure), nucleus accumbens, substantia nigra, and amygdala compared to AFR rats. Notably, litter-wise MS produced a completely different set of results; very few effects were detected in the young rats whereas measurement of peptides in adulthood revealed differences in a number of brain areas (**Figure 3B**). Higher levels were detected after litter-wise MS15 in the medial prefrontal cortex, VTA, and amygdala and lower levels were found in the substantia nigra after MS360 as compared to AFR rats. These results show that the effects induced by individual MS are immediate and detected in structures related to HPA axis function whereas the litter-wise MS produce changes that are detected several weeks after the MS period in areas related to reward and addiction and with a clear distinction between short and prolonged MS (see further "Short vs. Prolonged Maternal Separation").

MS-induced effects on oxytocin networks in rodents are dependent on protocol used and age of the analysed animals. Brief handling affected the number of neurons in the paraventricular nucleus compared to AFR (Todeschin et al., 2009). Prolonged MS for 180 min resulted in increased receptor binding in hypothalamus in 8- and 16-week-old rats but not in 5-week-old rats compared to AFR (Lukas et al., 2010). However, no differences in oxytocin levels were seen after MS360 compared to AFR in 3- or 10-week-old rats (Oreland et al., 2010). Pronounced differences in oxytocin levels were instead observed between rats exposed to MS15 and AFR rats. Both individual and litter-wise MS15 were associated with higher oxytocin levels in the hypothalamus and also in the pituitary gland (Oreland et al., 2010). The MS-induced differences in the hypothalamus and pituitary gland were attenuated in adult rats and not detectable in basal levels at 10 weeks of age (Oreland et al., 2010). The amygdala was also targeted by early environmental factors and in particularly after short separations. Oxytocin levels were lower after 15 min MS compared to AFR and low levels were also seen after litter-wise MS15 in adult rats, which indicates persistent alterations in basal oxytocin levels

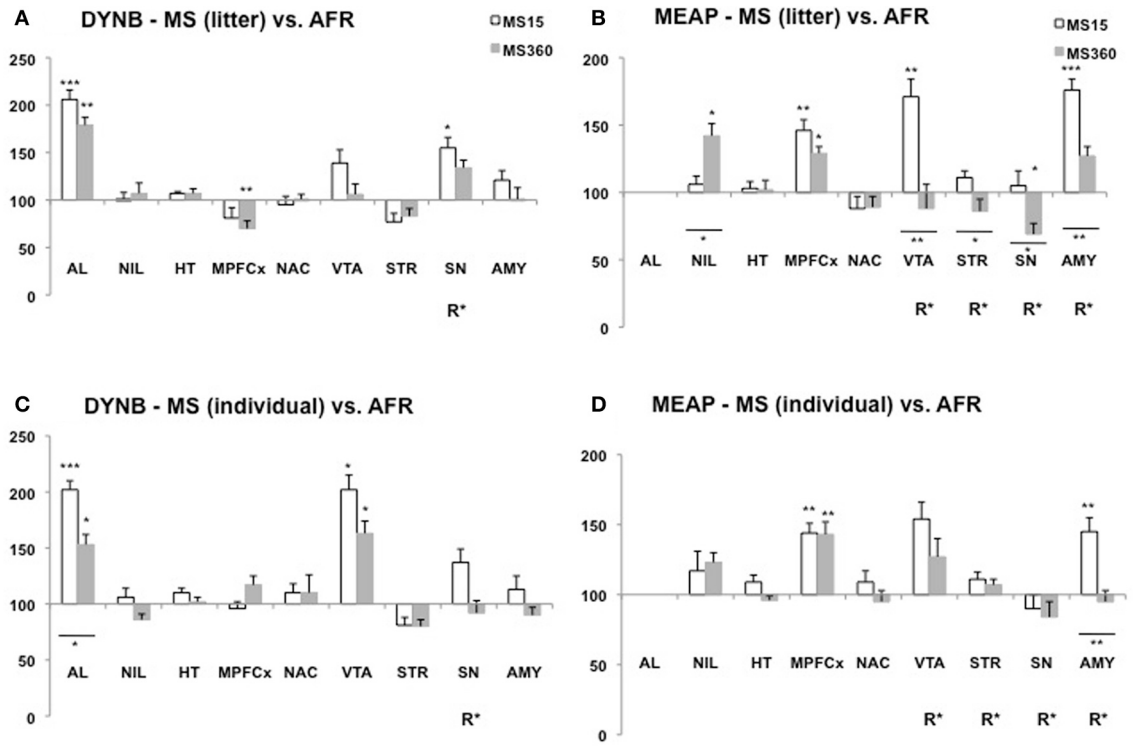

FIGURE 3 | Dynorphin B (DYNB) and Met-enkephalin-Arg^{6}Phe^7 (MEAP) in 10-week-old rats after exposure to different postnatal conditions. Immunoreactive (ir) levels of DYNB **(A,C)** and MEAP **(B,D)** in rats subjected to MS15 or MS360 in the litter-wise MS paradigm **(A,B)** and in the individual MS paradigm **(C,D)** were analysed at 10 weeks of age, seven weeks after the MS period. Values represent mean ± standard error of the mean and are shown as percent of the levels in animals subjected to animal facility rearing (AFR) conditions (data from Gustafsson et al., 2008). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. *Comparison with peptide levels in the AFR group. *Under a line, comparison between the MS15 and MS360 groups. R*, main effect of rearing environment, i.e., difference between all MS15 rats (litter-wise and individual separation) and all MS360 rats (litter-wise and individual separation). Abbreviations: AL, anterior lobe of the pituitary gland; NIL, neurointermediate lobe of the pituitary gland; HT, hypothalamus; MPFCX, medial prefrontal cortex; NAC, nucleus accumbens; VTA, ventral tegmental area; STR, dorsal striatum; SN, substantia nigra; AMY, amygdala.

in MS15 as compared to AFR (Oreland et al., 2010). Oxytocin levels in rats subjected to MS360 were similar to AFR (Oreland et al., 2010) and after MS180 no differences were observed in receptor binding in the amygdala as compared to AFR (Lukas et al., 2010). These studies provide evidence for a strong influence of early-life conditions on oxytocin. The peptide levels were similar after prolonged separations and AFR conditions whereas the exposure to MS15 was clearly different from AFR. Prolonged stress has been suggested to increase oxytocin activity to protect the animal from deleterious effects of stress (Carter et al., 2008). In that respect it is interesting to note that both AFR and MS360 had higher levels as compared to MS15. Considering the role of oxytocin in sensory stimulation such as warmth and touch (Uvnäs-Moberg et al., 1993; Ågren et al., 1995) it was of interest that the individual separations with repeated loss of tactile contact had a pronounced effect on the oxytocin in the amygdala.

MS also affects vasopressin but the results are not conclusive. High vasopressin mRNA levels have been reported in the paraventricular nucleus, but not in other parts of the hypothalamus, and in the bed nucleus of the stria terminalis in 5-week-old rats subjected to 180 min separation compared to AFR (Veenema and Neumann, 2009). However, no differences in vasopressin receptor binding in hypothalamus or amygdala were seen in adult rats (Lukas et al., 2010) and no differences in basal mRNA levels or vasopressin levels (Veenema et al., 2006) compared to AFR. With another protocol no differences were observed in vasopressin mRNA levels in the paraventricular nucleus after short 3 min individual separation compared to non-handled rats (Gabriel et al., 2005). Furthermore, no differences in basal vasopressin levels were observed in the pituitary gland, hypothalamus or amygdala at 3 or 10 weeks of age in rats subjected to either MS15 or MS360 compared to AFR (Oreland et al., 2010). Taken together, these studies indicate few or no differences between MS and AFR in basal levels of vasopressin.

Taken together, neuropeptide levels in rats exposed to MS clearly differ from levels in non-handled rats and rats housed according to conventional laboratory control conditions, i.e., AFR conditions. The most important take home message from the comparisons between rats subjected to short or prolonged MS vs. AFR is that the peptide levels differed between rats exposed to the shorter MS and AFR. In other words, the peptide levels in the conventional laboratory rat were different from those detected in rats reared under conditions similar to natural conditions. The outcome of MS will thus largely depend on whether AFR or short MS

is used as control group. It is also worth noting that the rats that were subjected to the putative most stressful experience, i.e., daily MS360 from both the dam and the littermates, had oxytocin levels comparative to the conventional laboratory rat. These results raise the question of how "normal" the conventional laboratory rat is.

SHORT VS. PROLONGED MATERNAL SEPARATION

To elucidate the impact of early-life risk factors and protective factors it is more relevant to compare the effects of repeated loss of maternal contact for extended periods, i.e., adverse conditions, with effects after shorter periods of maternal absence, i.e., beneficial conditions related to naturalistic mother-offspring interactions. This comparison is not confounded by differences in experimenter handling.

In studies where the animals were single-housed generally few differences were seen in DYNB levels between adult rats subjected to MS15 and MS360 (Ploj et al., 2003b; Gustafsson et al., 2007). Lower levels were, for example, detected in the substantia nigra and higher levels in the amygdala after MS360 (Ploj et al., 2003b) and these results were confirmed also in group-housed animals (Gustafsson et al., 2008). In the group-housed adult rats, the effects were more pronounced which again show that the housing conditions *per se* can affect peptide levels and thereby confound the outcome. A comparative study that assessed the effects of individual vs. litter-wise MS and also MS15 vs. MS360 at different ages revealed that individual and litter-wise MS resulted in similar effects on DYNB (Gustafsson et al., 2008). Differences between MS15 and MS360 (a significant effect of rearing, R, in **Figures 2** and **3**) was observed in the pituitary gland and the amygdala in young rats and in adult rats in the substantia nigra and the periaqueductal gray (Gustafsson et al., 2008).

Pronounced differences were seen in MEAP levels between MS15 and MS360 rats and it outcome was dependent on whether individual or litter-wise MS was used (Gustafsson et al., 2008). At 3 weeks of age, differences between MS15 and MS360 were noted in the pituitary gland, hypothalamus, striatum, and amygdala (**Figure 2**) and in adult rats in the VTA, striatum, substantia nigra, and amygdala (**Figure 3**). The effect seen shortly after the separation period was particularly evident after individual MS whereas the differences seen in adult rats were noted after litter-wise separation. These results are in agreement with findings in animals that were single-housed for two months where lower levels were seen after MS360 in the hypothalamus, medial prefrontal cortex, striatum, and also periaqueductal gray (Gustafsson et al., 2007). Taken together, generally lower MEAP levels were seen in the brain after prolonged MS in the litter-wise MS paradigm.

Early-life events may also affect enzymatic activity and thereby affect the levels of bioactive peptides. One enzymatic process that is interesting in terms of drug-induced reward, reinforcement, and addictive processes is the conversion of KOPR-acting DYNs into shorter peptides that act on the DOPR (Akil et al., 1998; Hallberg and Nyberg, 2003; Hallberg et al., 2005). An environmentally induced change in this enzymatic step will change the overall output of the proDYN system (see "Endogenous Opioids"). The impact of early-life conditions on enzymatic conversion or inactivation of peptides is poorly examined and currently addressed in our laboratory. Interestingly, we observe large differences in the rate of formation of DYN1-6 in animals previously exposed to the different early-life settings. These preliminary data indicate that early-life experiences can affect enzymatic processes and thereby change the final output from a peptide system.

Pronounced differences in oxytocin levels were observed between short and prolonged MS (Oreland et al., 2010). Oxytocin levels in the MS360 rats were lower in the pituitary gland but higher in the hypothalamus and amygdala compared to the MS15 rats at 3 weeks of age. Measurement in adult rats revealed that the difference between MS15 and MS360 was persistent in the amygdala but not in the other areas. As for vasopressin, no differences were found between MS15 and MS360 after individual or litter-wise MS, neither immediately after the MS nor in adulthood (Oreland et al., 2010).

Taken together, prolonged MS had pronounced effects on MEAP and oxytocin levels. The general finding was lower MEAP levels in adult animals that had been subjected to adverse early-life conditions. The individual and litter-wise separations clearly result in different effects on MEAP; more pronounced but short-lived immediate effects were seen after individual MS and distinct long-term effects in the litter-wise paradigm. As for oxytocin, exposure to the individual separation paradigm produced more pronounced effects and in the amygdala also more long-term changes. Less consistent effects were seen in DYNB levels but the reported difference between rats that were subjected to a proposed beneficial and stressful environment, respectively, early in life may have consequences for sensitivity to challenges later in life.

MULTIVARIATE ANALYSIS OF REARING CONDITIONS AND BASAL PEPTIDE LEVELS

To further investigate the relationship between short and prolonged MS, individual and litter-wise MS and peptide levels a multivariate data analysis was performed to analyse and illustrate the relations between all variables. The multivariate method partial least square discriminant analysis (PLS-DA) was used. PLS-DA is a regression extension of principal component analysis (PCA) and calculates the relationship between a Y-matrix (here rearing conditions) and an X-matrix (here peptide levels). The weights for the X-variables (in the analysis denoted w) indicate the importance of these variables, while the weights for the Y-variables (in the analysis denoted c) indicate which Y-variables are modeled in the respective PLS model dimensions. When these coefficients are plotted in a w × c plot, we obtain a picture showing the relationships between X and Y (Eriksson et al., 2006). This analysis included all peptide data (DYNB, MEAP, oxytocin, and vasopressin) from a large comparative study (Gustafsson et al., 2008; Oreland et al., 2010) in which the effects of daily MS15 and MS360 were assessed and compared with AFR and, in addition, examination of the influence of individual or litter-wise separations were investigated in both young and adult rats. The animals were all part of the same study with all experimental conditions exactly the same such as experimenter, laboratory housing conditions, and batch of rats (Gustafsson et al., 2008). The analysis was performed with all experimental groups including AFR

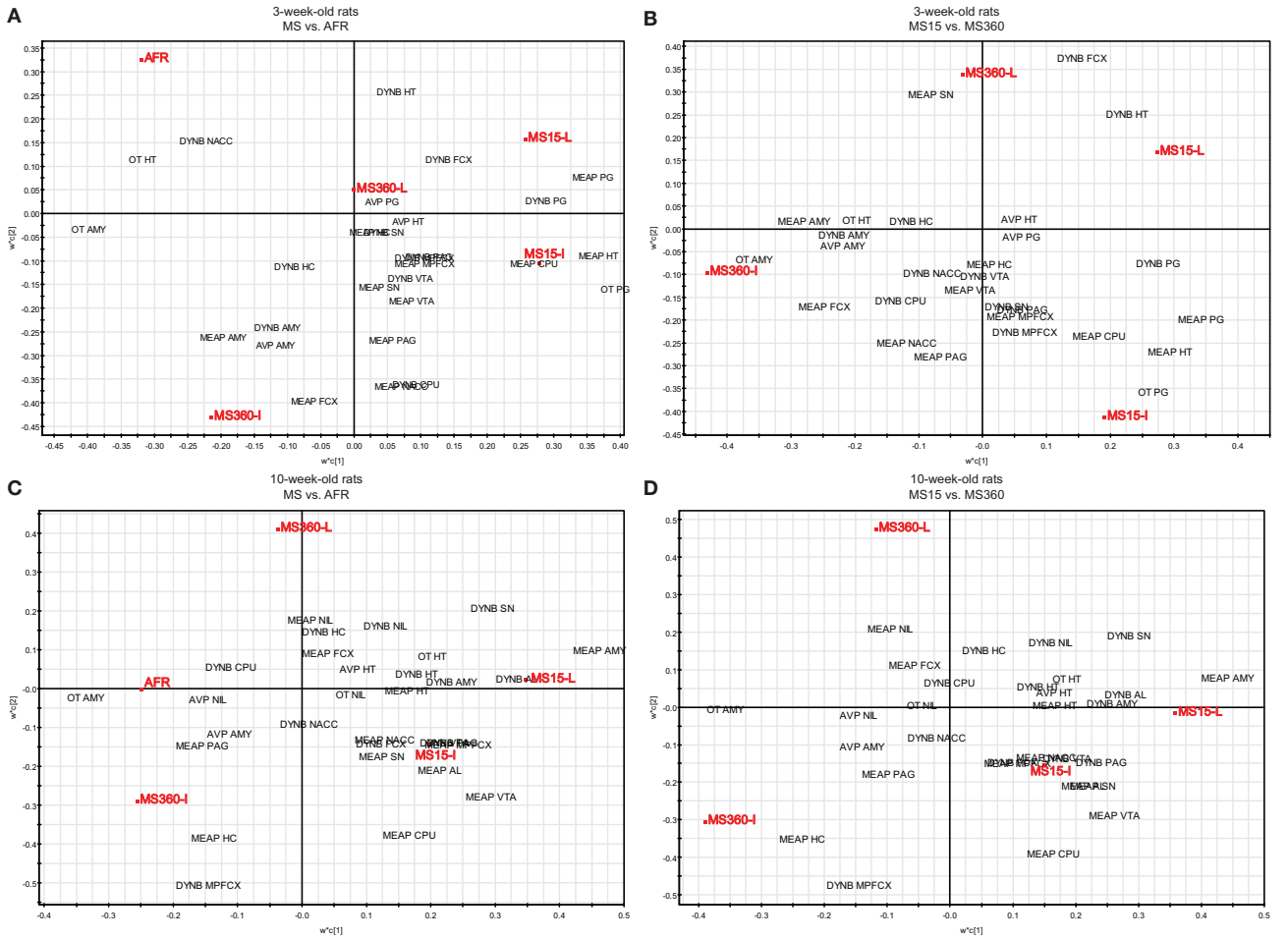

FIGURE 4 | Multivariate partial least square discriminant analysis (PLS-DA) of basal peptide levels. PLS-DA scatter plots of basal ir peptide levels in young (**A**, $R^2X(cum) = 0.218$, $R^2Y(cum) = 0.358$, $Q^2(cum) = 0.233$, two components and **B**, $R^2X(cum) = 0.223$, $R^2Y(cum) = 0.423$, $Q^2(cum) = 0.129$, two components) and adult (**C**, $R^2X(cum) = 0.204$, $R^2Y(cum) = 0.315$, $Q^2(cum) = 0.110$, two components and **D**, $R^2X(cum) = 0.201$, $R^2Y(cum) = 0.457$, $Q^2(cum) = 0.251$, two components) rats. Rearing conditions as Y and ir peptide levels as X. Data from Gustafsson et al., 2008; Oreland et al., 2010. Abbreviations: AL, anterior lobe of the pituitary gland; AMY, amygdala; AVP, vasopressin; CPU, caudate putamen = dorsal striatum; FCX, frontal cortex; HC, hippocampus; HT, hypothalamus; MPFCX, medial prefrontal cortex; NAC, nucleus accumbens; NIL, neurointermediate lobe of the pituitary gland; OT, oxytocin; PAG, periaqueductal gray; PG, pituitary gland; SN, substantia nigra; VTA, ventral tegmental area.

(**Figures 4A** and **C**) and without AFR with only those groups exposed to the same handling included (**Figures 4B** and **D**). In the three-week old animals (**Figure 4A**) there is a clear separation between AFR and individually separated MS360 (in the left quadrants) and MS15 rats (in the right quadrants). Furthermore, individually separated MS15 rats load in the lower right quadrant, separated from litter-wise separated MS15 and MS360 rats. When the AFR group was excluded from the analysis (**Figure 4B**), all four MS conditions separate from each other, with the individually separated MS360 rats being the most extreme group. In adult animals (**Figure 4C**), AFR rats and MS360 rats load in the left quadrants and separate from the MS15 rats. Moreover, in the right quadrants AFR rats load in-between litter-wise and individually separated MS360 rats. Separation condition (i.e., litter-wise or individual) seems to be of less importance for adult MS15 rats. When the AFR group was excluded from the analysis (**Figure 4D**), a similar patter as in the young animals appear with a clear separation between litter-wise and individually separated MS360 rats, and MS15 rats. These results confirm the notion that prolonged MS is similar to the AFR condition whereas the MS15 clearly separates from both the MS360 and AFR groups. The results also show that the differences are persistent into adulthood and, finally, they confirm the notion from other analyses that short litter-wise MS, which resemble natural conditions, are clearly separated from the AFR condition.

IMPLICATIONS FOR ALCOHOL USE DISORDERS (AUD)

Evidence for a link between ethanol and endogenous opioids have been shown in numerous studies. Ethanol-induced opioid activation is suggested to contribute to ethanol reward and reinforcement (Nylander and Silberring, 1998; Barson et al., 2009). Opioid receptor antagonists attenuate the ethanol-induced increase in extracellular dopamine (Benjamin et al., 1993) and reduce ethanol consumption in laboratory animals (Ulm et al.,

1995) and in humans (O'Malley et al., 1992; Volpicelli et al., 1995). This effect may relate to diminished ethanol-induced reward and today naltrexone is used in the treatment of AUD. Endogenous opioids have also attracted interest in terms of the etiology of AUD and for the neurobiological basis for individual differences in the propensity to develop AUD (Nylander et al., 1994; Gianoulakis, 1996; Nylander and Silberring, 1998; Modesto-Lowe and Fritz, 2005; Trigo et al., 2010). However, several possible mechanisms have been proposed for the involvement of opioids in the vulnerability/resilience for AUD (Van Ree et al., 1999; Gianoulakis, 2004; Oswald and Wand, 2004; Sanchis-Segura et al., 2005; Drews and Zimmer, 2010). One line of evidence suggests that an inherent opioid deficiency with low levels of endogenous opioids leads to enhanced ethanol consumption that stimulates opioid activity and compensate for the deficiency (Trachtenberg and Blum, 1987). Another hypothesis assumes that vulnerable individuals inherit or acquire an excess of opioid activity that leads to increased ethanol consumption (Reid et al., 1991). In addition it has been suggested that the vulnerability for increased ethanol consumption is determined by individual differences in sensitivity of the opioid system to ethanol (Gianoulakis, 1996). Here we extend the ethanol-opioid link and suggest a link between early-life environment, opioids, and ethanol consumption.

ASSOCIATION BETWEEN BASAL PEPTIDE LEVELS AND ETHANOL CONSUMPTION AFTER MS

In agreement with other studies (Weinberg, 1987; Hilakivi-Clarke et al., 1991; Huot et al., 2001; Ploj et al., 2003a; Jaworski et al., 2005; Gustafsson and Nylander, 2006) we have shown that adult rats previously subjected to postnatal prolonged MS have propensity for high ethanol intake and preference. Adult male MS360 rats had a higher voluntary ethanol consumption (Ploj et al., 2003a) and higher preference for 20% ethanol (Gustafsson and Nylander, 2006) in continuous access paradigms and higher ethanol intake and preference in an intermittent ethanol consumption paradigm (Daoura et al., 2011) compared to MS15 that were reared according to naturalistic conditions. These differences may relate to long-term behavioral differences induced by the respective short and prolonged repeated postnatal litter-wise separations from the dam. The adult behavioral profile of MS360 rats includes higher exploration, higher risk-assessment, and somewhat higher risk-taking behavior (Roman et al., 2006), which resembles that of ethanol-preferring AA rats (Roman et al., 2007, 2012). There are so far no indications that the high ethanol consumption in MS360 rats is a result of anxiety-related behaviors; the MS360 rats show anxiety-like behavior at three weeks of age but not in adulthood (Ploj et al., 2002; Roman et al., 2006).

Neurobiological differences between MS15 and MS360 rats may also contribute to the differences in ethanol consumption patterns. The findings so far do not support a clear association between ethanol intake and basal levels either of DYNB, oxytocin or vasopressin in animals subjected to different early-life conditions. However, as for the opioid peptide MEAP a series of studies using different MS paradigms have provided evidence for an association between the MS-induced effects on basal levels and ethanol consumption. These studies revealed that the difference in voluntary ethanol intake behavior between MS15 and MS360 was observed in one specific MS experimental set-up, i.e., the litter-wise MS paradigm and only in male rats (Ploj et al., 2003a; Gustafsson and Nylander, 2006; Daoura et al., 2011). In this set-up, adult male MS30 rats also had lower MEAP levels in brain areas related to reward, motivation, and addiction processes (Gustafsson et al., 2008). In contrast, there were no differences in the ethanol consumption (Roman et al., 2004; Gustafsson et al., 2005) and only minor neurobiological changes in these brain areas in adult female rats (Gustafsson et al., 2005). When young rats were given access to ethanol throughout adolescence there was no difference in ethanol intake between MS15 and MS360 (Daoura et al., 2011) and in young rats there were no differences in basal MEAP levels (Gustafsson et al., 2008). Finally, when the rats were subjected to individual MS instead of litter-wise MS the ethanol consumption in adult rats was similar regardless of rearing according to the MS15 or MS360 condition (Oreland et al., 2011) and only minor differences in MEAP were seen in reward-related brain areas (Gustafsson et al., 2008). Individual MS resulted in changes in areas associated with HPA axis activity in young rats but these differences between MS15 and MS360 were not persistent (Gustafsson et al., 2008) and presumably had little relevance for ethanol consumption behavior in the adult rat.

ASSOCIATION BETWEEN EARLY-LIFE CONDITIONS AND ETHANOL-INDUCED EFFECTS

The first responses to ethanol are important determinants for the individual drug-taking behavior (Schuckit et al., 2004). It is therefore of interest to examine whether individuals exposed to early-life risk respond differently to ethanol. An environmentally induced alteration in ethanol response during the initiation and habituation of ethanol consumption may relate to the individual liability to go from a controlled, habitual drinking to an uncontrolled and compulsive use.

It is well-established that endogenous opioids are involved in ethanol-induced actions although the exact mechanisms are not clear (Van Ree et al., 2000; Gianoulakis, 2004; Spanagel, 2009; Trigo et al., 2010). A number of studies describe the effects of ethanol on opioids but the majority of these studies examine effects after forced drinking (Schulz et al., 1980), liquid diet (Seizinger et al., 1983), injection procedures (Lindholm et al., 2000), or inhalation in a vapour chamber (Zapata and Shippenberg, 2006) and do not address the effects of long-term voluntary ethanol drinking in animals without genetic preference for ethanol. As for DYNB, a free choice between ethanol and water in adulthood resulted in enhanced levels in the anterior lobe of the pituitary gland, hypothalamus, medial prefrontal cortex, and substantia nigra whereas ethanol-induced low levels were seen in the neurointermediate lobe of the pituitary gland (Gustafsson et al., 2007). These ethanol-induced effects were similar in rats subjected to short and prolonged separations. However, a negative correlation between the intake of 20% ethanol and DYNB levels was observed in the amygdala in the MS360 rats but not MS15 rats (Gustafsson et al., 2007) which may relate to differences in ethanol intake in these rats; DYNs mediate aversive actions (Akil et al., 1998) and low DYNB levels in animals with a high ethanol intake may relate to their propensity to consume more ethanol.

Large differences were noted in the ethanol-induced effects on DYN1-6 depending on early-life environmental history (previously unpublished results; **Figure 5**). The DYN1-6 levels were measured in rats that were subjected to daily MS15 or MS360 and then given access to a free choice between ethanol and water for eight weeks in adulthood. The tissue levels of DYN1-6 were measured in the same animals as used for analysis of DYNB and MEAP [for detailed experimental design, see (Gustafsson et al., 2007)]. Voluntary ethanol intake affected DYN1-6 in several brain areas and in the pituitary gland (**Figure 5**) and the effects was more pronounced than those seen in the other opioid peptides (Gustafsson et al., 2007). An overall effect of ethanol was noted in the anterior lobe of the pituitary gland, neurointermediate lobe of the pituitary gland, hypothalamus, nucleus accumbens, striatum, hippocampus, amygdala, and periaqueductal gray. Interestingly, a statistically significant interaction between rearing condition (MS15 vs. MS360) and ethanol (ethanol vs. water) was also seen in several areas: in the neurointermediate lobe of the pituitary gland, frontal cortex, medial prefrontal cortex, striatum, hippocampus, and in the VTA (**Figure 5**). These results show that long-term ethanol drinking has a pronounced impact on the DYN1-6 levels and that the ethanol-induced effects in adult rats are dependent on the previous exposure to either postnatal MS15 or MS360. The effects on DYN1-6 were clearly distinct from the effects on DYNB indicating different effects on bioactive peptides derive from the same prohormone. Considering the pronounced effects described herein on DYN1-6 and the opposite effects induced by KOPR and DOPR ligands on networks involved in reward and motivation actions (Spanagel et al., 1992; Akil et al., 1998; Nylander and Silberring, 1998; Van Ree et al., 1999) further studies are warranted on different proDYN-derived peptides.

Long-term voluntary drinking of ethanol in adulthood was shown to differentially affect MEAP levels in rats previously exposed to different early-life conditions. The ethanol-induced effects were more pronounced in MS360 rats compared to the MS15 rats in several brain areas: the hypothalamus, medial prefrontal cortex, striatum, substantia nigra, and periaqueductal gray (Gustafsson et al., 2007). These results indicate that animals exposed to early adversity have an enhanced sensitivity to the effects of ethanol. Altered sensitivity to ethanol has been described in studies using ethanol-preferring rats such as the P rats (Li et al., 1998) and AA rats (Nylander et al., 1994). These results indicate similar phenotypes after selective breeding and exposure to environmental adversity. Opioids stimulate ethanol intake and it is suggested that ethanol drinking activates opioids that further trigger ethanol intake (Barson et al., 2009). A higher ethanol-induced activation of ENKs in the MS360 rats may relate to risk for an increased liability to escalate drinking.

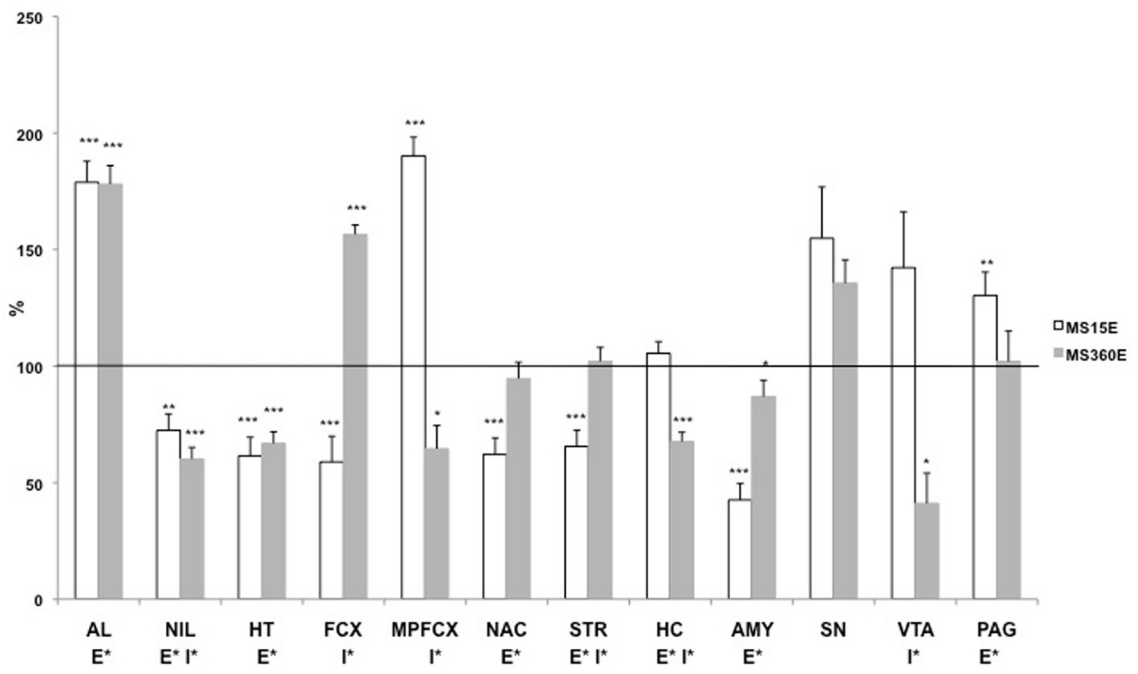

FIGURE 5 | The DYN1-6 levels in the pituitary gland and different brain areas in adult ethanol-drinking rats after exposure to different postnatal conditions. The rats have been exposed to either MS15 or MS360 during the first three postnatal weeks and from ten weeks of age they were given free access to ethanol for eight weeks before decapitation. The levels represent immunoreactive (ir) DYN1-6 (Leu-enkephalin-Arg^6) levels expressed as percent of the basal ir levels in water-drinking MS15 and MS360 rats, respectively. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, compared to water-drinking rats. E*, main effect of ethanol, i.e., difference between all ethanol-drinking rats (MS15 and MS360) compared to water-drinking rats (MS15 and MS360); I*, interaction effect, i.e., different effect of ethanol in the MS15 and the MS360 rats (Two-Way analysis of variance). Abbreviations: AL, anterior lobe of the pituitary gland; NIL, neurointermediate lobe of the pituitary gland; HT, hypothalamus; MPFCX, medial prefrontal cortex; NAC, nucleus accumbens; VTA, ventral tegmental area; STR, dorsal striatum; SN, substantia nigra; AMY, amygdala.

Ethanol-induced effects in animals were examined in rats that were subjected to the same experimental conditions as in the study by Gustafsson et al. (2007). Voluntary ethanol drinking for eight weeks resulted in lower oxytocin levels and higher vasopressin levels in the neurointermediate lobe of the pituitary gland. In the hypothalamus ethanol drinking resulted in higher oxytocin levels whereas the vasopressin levels were not affected (unpublished results). However, these ethanol-induced effects on oxytocin or vasopressin were similar in rats that had been subjected to MS15 and MS360 the results provide no evidence for associations between either oxytocin or vasopressin and environmentally induced changes in ethanol consumption.

MULTIVARIATE ANALYSIS OF REARING CONDITIONS AND PEPTIDE LEVELS IN WATER- AND ETHANOL-DRINKING RATS

PLS-DA (for details, see section "Multivariate Analysis of Rearing Conditions and Basal Peptide Levels") was again used in order to investigate possible relationships between rearing conditions, ethanol-induced effects, and peptide levels. Here data from a study in which opioid peptides (DYNB, MEAP, and DYN1-6), oxytocin, and vasopressin were assessed in brain areas and in the pituitary gland in rats that were given access to either water or a free choice between ethanol and water for eight weeks. The animals were all part of the same study (Gustafsson et al., 2007) with the same experimenter handling, laboratory housing conditions, and batch of rats from the supplier. The results show that the respective groups clearly load in separate quadrants with a separation between water-drinking animals in the left quadrants and ethanol-drinking rats in the right quadrants (**Figure 6**). Ethanol consumption in the MS360 rats changes the location from the lower to the upper quadrant whereas the opposite is seen for the MS15 groups, which indicates that different peptide profiles are affected in these experimental groups. These results confirm the notion that ethanol intake affects endogenous opioids differently depending on early-life environment, i.e., exposure to MS15 or MS360.

EARLY-LIFE CONDITIONS AND THE RESPONSE TO EXOGENOUS OPIOIDS

Early-life rearing conditions have been shown to change the effects of opioid receptor agonists and antagonists. Rats subjected to prolonged MS had an altered sensitivity to morphine (Kehoe and Blass, 1986; Kalinichev et al., 2001a,b) and enhanced naltrexone-induced suppression of sucrose consumption as compared to non-handled rats (Michaels and Holtzman, 2007). These results raised the question whether also the effects of naltrexone on ethanol consumption would be affected by early-life conditions. An increasing number of reports describe large individual

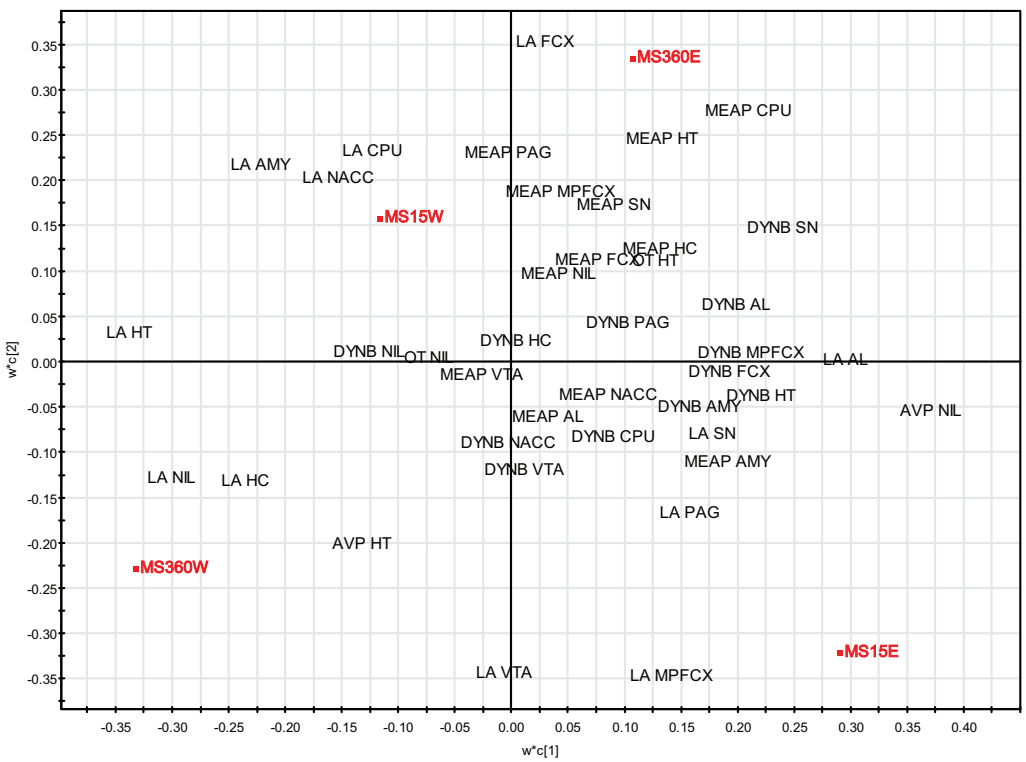

FIGURE 6 | Multivariate partial least square discriminant analysis (PLS-DA) of peptide levels in water- and ethanol-drinking rats. PLS-DA scatter plot of the two first components ($R^2X(cum) = 0.269$, $R^2Y(cum) = 0.706$, $Q^2(cum) = 0.555$). Rearing conditions as Y and ir peptide levels as X. Data from (Gustafsson et al., 2007). Abbreviations: AL, anterior lobe of the pituitary gland; AMY, amygdala; AVP, vasopressin; CPU, caudate putamen = dorsal striatum; FCX, frontal cortex; HC, hippocampus; HT, hypothalamus; MPFCX, medial prefrontal cortex; LA, Leu-enkephalin-Arg^6 = DYN(1–6); NAC, nucleus accumbens; NIL, neurointermediate lobe of the pituitary gland; OT, oxytocin; PAG, periaqueductal gray; SN, substantia nigra; VTA, ventral tegmental area.

variations in the effects of naltrexone in AUD patients (Kiefer et al., 2008; Spanagel and Kiefer, 2008) and pharmacogenetic studies have revealed that genetic factors contribute to individual differences in the ability of naltrexone to reduce ethanol intake (Monterosso et al., 2001; Rubio et al., 2005; Ray et al., 2010; Roman and Nylander, 2005; Moffett et al., 2007; Barr, 2011). For example, several reports have shown that MOPR polymorphism affect the response to naltrexone (Oslin et al., 2003; Mague and Blendy, 2010).

In a recent study it was confirmed that the response to naltrexone differs depending on early-life environmental conditions (Daoura and Nylander, 2011). Adult rats previously exposed to daily MS360 decreased their voluntary ethanol consumption after administration of naltrexone whereas animals subjected to MS15 did not respond (Daoura and Nylander, 2011). These results provided evidence that ethanol-drinking rats with a history of adverse early environmental experiences responded well to naltrexone, whereas rats reared in an environment related to positive behavioral consequences did not benefit from naltrexone treatment. A plausible explanation for different effects of naltrexone is the different ethanol-induced effects in MS15 and MS360 rats; MS360 rats had higher MEAP levels after voluntary ethanol consumption, whereas no ethanol-induced increase in MEAP was found in the MS15 rats (Gustafsson et al., 2007). It is therefore likely that naltrexone abolished the effect of ethanol-induced opioid activation, thereby attenuating the ethanol-induced reward and reducing ethanol intake in the MS360 rats. Similarly, the lack of ethanol-induced activation of opioids in MS15 rats may explain the poor efficacy of naltrexone in these rats. These results highlight the importance of not only considering genetic factors but also early environmental factors when identifying subtypes of AUD patients that respond well to naltrexone treatment.

Taken together, the summarized results provide evidence for a link between early-life experiences, opioids, and adult voluntary ethanol consumption. Studies so far indicate that there is no clear relation between DYNB, oxytocin or vasopressin, and ethanol consumption while there is an association between basal levels of MEAP and ethanol consumption in animals exposed to MS. In addition, differences in response to voluntary ethanol drinking and challenge with opioid agonists and antagonists after exposure to different early-life conditions have been described. Adult male rats exposed to MS360 are characterized by altered risk-taking behavior and propensity for high ethanol intake had lower central levels of MEAP and a more pronounced ethanol-induced increase in MEAP levels and they also responded with decreased ethanol consumption after naltrexone. Opioid deficiency and an enhanced sensitivity to the effects of ethanol on opioids have been proposed to be part of the neurobiological basis for increased vulnerability to addiction. Persistent changes in central ENK networks, caused by early-life experiences, may therefore contribute to the liability to go from the controlled drinking to excessive drinking and the compulsive use seen in AUD.

SUMMARY AND FUTURE DIRECTIONS

The effects described herein are summarized from studies that employed rodent MS procedures to simulate different early-life settings. There are also other early-life settings, for example enrichment paradigms that are known to affect later drug consumption patterns, such as amphetamine self-administration (Bardo et al., 2001). It has also been shown that the rewarding effects of cocaine are inversely related to the degree of enrichment (Zakharova et al., 2009). Enrichment paradigms are not accounted for here and to our knowledge less is known about the effects of enrichment on peptide networks. In the present article it is shown that rearing in different early-life environments have pronounced impact on basal peptide levels, in enzymatic activity and in drug-induced effects on neuropeptides. Some effects on the peptides were immediate and seen already in the young animals and were either short lasting and attenuated, "normalized," during adolescence or persisted into adulthood. However, the altered levels early in life point to affected peptide circuits and even though no differences could be detected in basal levels in adulthood the system may still be more sensitive to challenges later in life. Other effects appeared later in adulthood, which indicate that early environmental factors can affect neuronal networks or processes involved in peptide ontogenesis, change the developmental pattern and thereby not be detectable in basal levels until adulthood.

The summarized results show that the most pronounced effects of early-life exposure were on oxytocin and MEAP. This observation is not surprising considering that these peptide systems continue to develop and mature after birth (McDowell and Kitchen, 1987; Buijs, 1992; Lipari et al., 2001). DYN (Leslie and Loughlin, 1993) and vasopressin (Lipari et al., 2001) also continue to develop after birth but they are more fully developed at birth as compared to MEAP and oxytocin. These peptides may therefore be less sensitive to postnatal manipulations and fewer changes in basal levels of DYNB and vasopressin were also noted. However, as already mentioned, the effects of exposure to a risk environment may not be seen in basal levels but appear when the individual encounters challenge later in life such as stress, trauma, or risk consumption of drugs of abuse. DYN, for example, have been suggested to be involved in anti-reward systems in the brain (Bruijnzeel, 2009; Wee and Koob, 2010) and environmentally induced changes in DYN networks may come into play after long-term drug consumption in the addictive and withdrawal states.

Considering the vast number of physiological functions that have been described for the endogenous opioids (e.g., Van Ree et al., 1999; Kieffer and Gaveriaux-Ruff, 2002; Przewlocki, 2002; Trigo et al., 2010; Bodnar, 2011) it is evident that long-term changes in opioid functioning induced by early-life experiences will have extensive consequences for the individual. The results presented here provide evidence for a link between the early-life environment, endogenous opioids, and adult ethanol consumption. Exposure to an early-life setting associated with disturbed interactions between the caregiver and the offspring result in long-term changes in basal levels of the ENK peptide MEAP and altered response to ethanol in adult rats. Dysfunctional opioid networks and altered ethanol-induced response in rats exposed to early-life stress may relate to their high ethanol consumption and also to the high efficacy of naltrexone in reducing ethanol intake. Endogenous opioids are therefore relevant to further study

as putative mediators of environmentally induced vulnerability or resilience to AUD.

Altered oxytocin levels were seen in young rats in the hypothalamus, pituitary gland and the amygdala and the repeated loss of tactile contact during the separations in the individual MS paradigm resulted in persistent effects in the amygdala. These results show that oxytocin is a target for early-life influence and show the importance of early-life tactile contact for normal oxytocin development. The differences between basal oxytocin levels in rats subjected to a risk and a preventive environment, respectively, were not associated with differences in ethanol consumption (Oreland et al., 2010) and the ethanol-induced effects were similar.

However, considering the reports of links between oxytocin, early-life behavior and mental health (Panksepp, 1992; Meyer-Lindenberg, 2008) it is likely that environmentally induced changes in oxytocin functioning will affect later behavior. Oxytocin has anxiolytic effects (McCarthy et al., 1996; Bale et al., 2001; Ring et al., 2006), is involved in stress-coping behavior (Neumann, 2002; Ebner et al., 2005) and in fear processes (Viviani and Stoop, 2008) and early-life changes in the hypothalamus and in the amygdala may contribute to altered behavior and sensitivity to other challenges. Oxytocin functioning, especially in the amygdala has been related to autism (Bartz and Hollander, 2008; Meyer-Lindenberg, 2008) and an altered oxytocin function as a consequence of early-life experiences may therefore be of interest in the etiology of autism spectrum disorders. The results summarized herein show that MS may be utilized as an experimental model in further studies of the role of oxytocin in the early environmental impact on brain function, behavior, and pathology.

The advantage with animal experimental studies as those described in the present article is that the early environment can be manipulated and that the neurobiological consequences can be studied in detail under controlled conditions. The interactive effects of rearing conditions and later challenges can be studied. Of relevance for AUD is the possibility to study the effects of ethanol consumption in individuals with different histories of early-life experiences and distinguish between long-term effects caused by environmental factors in juveniles from the effects of ethanol consumption in adolescents. The interactive effects of early-life experiences and genotype can be studied using rodents with genetic ethanol preference such as described in studies of the ethanol-preferring AA rats (Roman et al., 2003, 2005). The epigenetic mechanisms underlying early-life impact on the brain can be delineated. Genes of interest for vulnerability or resilience to AUD can be identified in humans and the interactions with environmental factors can be further studied in genetically modified animals. Along with the development of more advanced gene technology we can insert/delete/modify genes of interest in rodents and examine the detailed mechanisms of environmentally induced alterations in gene activity in neurobiological and behavioral studies.

In conclusion, experimental studies have provided evidence that early-life events cause long-term alterations in neuropeptide circuits, especially oxytocin and the opioid peptide MEAP. The results indicate a link between early-life rearing conditions, opioid levels, and ethanol consumption and show that the ethanol-induced effects and the treatment with opioid antagonists later in life are dependent on early-life experiences. Endogenous opioids are therefore of interest to further study in the early-life impact on individual differences in vulnerability to AUD and treatment outcome. It is also evident from the studies presented herein that it is highly important to be particulate in the design and description of experimental conditions in MS studies. MS-induced effects on neuropeptides were dependent on the length of separations, whether the rat pups are kept together or placed individually during the separations, on the age when the effects are analysed and whether males or females were examined. Today there are a number of different protocols in use and provided that the experimental conditions are clearly described and motivated the plethora of protocols is not an obstacle but rather an advantage; they facilitate the choice of an appropriate experimental design depending on the question addressed.

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