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Neurobiology of the incubation of drug craving

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Abstract

It was suggested in 1986 that cue-induced drug craving in cocaine addicts progressively increases over the first several weeks of abstinence and remains high for extended periods. During the last decade, investigators have identified an analogous incubation phenomenon in rodents, in which time-dependent increases in cue-induced drug seeking are observed after withdrawal from intravenous cocaine self-administration. Such an incubation of drug craving is not specific to cocaine, as similar findings have been observed after self-administration of heroin, nicotine, methamphetamine, and alcohol in rats. In this review, we discuss recent results that have identified important brain regions involved in the incubation of drug craving, as well as evidence for the underlying cellular mechanisms. Understanding the neurobiology of the incubation of drug craving in rodents is likely to have significant implications for furthering our understanding of brain mechanisms and circuits that underlie drug craving in human addicts.

Introduction

Relapse to drug use in humans can occur after prolonged abstinence [1] and is often precipitated by exposure to drug-associated cues that provoke drug craving [2] (Glossary). In an attempt to account for this persistent relapse, it was hypothesized that cue-induced cocaine craving progressively increases over the first weeks of abstinence and remains high over extended periods [3]. An analogous incubation phenomenon, termed incubation of drug craving [4], was subsequently identified in rats, based on observations that time-dependent increases in cue-induced cocaine or heroin seeking occurred after drug withdrawal [4–6].

Incubation of craving has also been observed in rats with a history of methamphetamine [7], alcohol [8], nicotine [9], or oral sucrose self-administration [10, 11] (Fig. 1) (see ref. [12] for an early demonstration of “incubation of food craving”). Such a phenomenon has been demonstrated using several established procedures to assess the motivational impact of reward cues and cue-induced relapse to drug seeking [13–15], including extinction [16–19], cue-induced reinstatement [4, 8, 9, 20, 21], and acquisition of a new response [22] behavioral procedures.

In humans, drug craving is not only induced by drug cues but also by re-exposure to the drug itself or exposure to stress [23, 24]. These stimuli are known to cause reinstatement of drug seeking in non-human laboratory animals [25–29], however, unlike drug cues,

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evidence for "incubation" of the rat's response to drug re-exposure (often termed drug priming) is mixed, with reports of time-dependent increases [30], decreases [31] or no change [18, 32] in cocaine-priming-induced reinstatement of drug seeking. However, there is evidence for time-dependent changes in intermittent-footshock-stress-induced reinstatement after withdrawal from heroin or cocaine [6, 17]. This "stress incubation" effect, however, was not observed in reinstatement of methamphetamine seeking by the pharmacological stressor yohimbine [7].

Several factors influence the magnitude of incubation of cocaine craving in rodents, including age, housing conditions, exercise, and sex hormones. Incubation of craving was observed to be weaker in adolescent rats than in adult rats [33]. Housing in an enriched environment during abstinence was found to decrease the magnitude of incubation of craving for cocaine in rats [34, 35] (see also [36] for similar results observed for the reinstatement of sucrose seeking behavior). Exercise during abstinence decreased extinction responding and cue-induced reinstatement of cocaine seeking in rats [37] under self-administration training conditions that lead to incubation of cocaine craving [38]. Finally, incubation of cocaine craving was more pronounced in female rats during estrus than in non-estrus females or males [39].

In this review, we discuss recent studies which have investigated neurobiological mechanisms underlying the incubation of drug craving. Earlier studies on the incubation of cocaine craving have been covered in previous reviews (see [40]). Because of space limitations, we do not review correlational studies in which changes in gene expression and proteins were assessed in the absence of experimental manipulations to assess a causal role of the molecular changes in incubation of craving for cocaine [41–45] or other drugs [9, 46–49].

Brain regions and molecular mechanisms involved in the incubation of drug craving

Many studies using animal models indicate that the mesocorticolimbic dopamine system and the nigrostriatal dopamine system both contribute to cue-induced cocaine seeking [14, 50, 51] and other behavioral effects of cocaine, including cocaine reward [52, 53]. The mesocorticolimbic dopamine system is comprised of cell bodies in the ventral tegmental area [VTA] that project to many forebrain areas, including the medial prefrontal cortex [mPFC], nucleus accumbens [NAc], and amygdala, while the nigrostriatal dopamine system is comprised of cell bodies in the substantia nigra [SNA] that project to the dorsal striatum.

Below, we first describe results from four studies in which investigators used classical neuropharmacological and electrophysiological methods to study the role of the NAc, mPFC, dorsolateral striatum, and basolateral amygdala (BLA) in the incubation of cocaine craving. We then describe studies that have identified roles for extracellular signal-regulated kinase (ERK), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), glutamate, and GluA2-lacking AMPA receptors (AMPARs) in different components of the mesocorticolimbic dopamine system in incubation of drug craving (Fig. 2).

Brain regions

Incubation of cocaine craving, as assessed in extinction tests after 1 or 30 withdrawal days, is associated with time-dependent increases in cue-induced neuronal activation (as assessed by in vivo electrophysiology in behaving rats) in the core of the NAc [16]. This incubation is also associated with time-dependent increases in cue-induced neuronal activation in the mPFC (as assessed by ERK phosphorylation, a common marker of neuronal activity) [54].

Subsequent experiments demonstrated that neuronal activity in the ventral but not dorsal mPFC likely plays a causal role in incubation of cocaine craving. In this study, ventral but not dorsal mPFC pharmacological inhibition (achieved using a mixture of $GABA_A$ and $GABA_B$ agonists) on withdrawal day 30 decreased cocaine seeking, while ventral but not dorsal mPFC activation (via a mixture of $GABA_A$ and $GABA_B$ antagonists) on withdrawal day 1 increased cocaine seeking [54].

When an "incubation" procedure [18] was combined with a reconsolidation procedure [55], it was reported that inhibition of the immediate early gene, Early Growth Response Protein 1 (Egr1, previously known as *Zif268*) in the BLA prior to exposure to cocaine-associated cues after 3 withdrawal days decreased the expression of incubation of cocaine craving up to 30 days after withdrawal from the drug [19]. These data suggest that the activity of Egr1 in the BLA is critically involved in mediating reconsolidation of memories of cues previously paired with cocaine self-administration. However, the degree to which activity in the BLA is involved in the incubation of cocaine craving remains unknown. Specifically, incubation of cocaine craving is observed in the absence of any cue exposure between drug self-administration training and late withdrawal tests [40]. Therefore, it is unlikely that a reconsolidation process for the putative cue memory after memory reactivation plays a role in incubation of cocaine craving.

The role of the dorsolateral striatum in the incubation of cocaine craving was assessed in a study in which this brain site was reversibly inactivated in rats (via a mixture of $GABA_A$ and $GABA_B$ agonists) prior to extinction tests after 1, 14, and 60 days of withdrawal from limited or extended access to cocaine [56]. Dorsolateral striatum inactivation decreased cocaine seeking independent of cocaine exposure history or withdrawal period duration [56], suggesting that this brain site does not play a unique role in incubation of cocaine craving. These data, however, confirm previous results on the role of the dorsolateral striatum in cue-induced reinstatement of drug seeking [50, 57, 58].

In summary, results from studies using electrophysiological and neuropharmacological methods suggest a role of the ventral mPFC and NAc, but not dorsolateral striatum, in the incubation of cocaine craving. The BLA is known to be involved in the reconsolidation of memories of cues previously paired with drug self-administration; however, the degree to which this brain area contributes to the incubation of drug craving remains currently unknown. In the remaining sections of the review, we discuss results from studies that have combined molecular and neuropharmacological methods to further investigate the role of the NAc in the incubation of cocaine craving. We also discuss studies that have indicated important roles for the central amygdala (CeA) and the VTA in this incubation.

A role for ERK and glutamate signaling pathways in the central amygdala

ERK is a component of the mitogen-activated protein kinase (MAPK) intracellular signaling pathway and its activation plays an important role in synaptic plasticity mechanisms underlying learning and memory [59, 60]. Cocaine and other drugs increase ERK activity in components of the mesolimbic dopamine system, and ERK activity contributes to cocaine psychomotor sensitization and reward, as well as consolidation and reconsolidation for memories of cocaine cues [61, 62]. ERK activation also mediates some of the physiological effects of both BDNF and GDNF [63], including those related to the effects of exogenous BDNF and GDNF administration on drug-related behaviors [64–67] (see below).

Time-dependent increases in cocaine seeking after withdrawal are correlated with time-dependent increases in ERK activity in the CeA but not the BLA [68]. Furthermore, pharmacological inactivation of CeA but not BLA ERK activity decreased cocaine seeking after 30 withdrawal days. Additionally, stimulation of CeA ERK activity by the glutamate

agonist NMDA on withdrawal day 1 increased cocaine seeking, an effect reversed by pharmacological inhibition of ERK activation. These data establish a causal role of CeA ERK in the expression of incubation of cocaine craving and suggest that glutamate signaling enhances the responsiveness of CeA ERK to cocaine cues after prolonged withdrawal. As mentioned above, stimulation of CeA ERK activity by NMDA increased cocaine seeking on withdrawal day 1; additionally, blockade of ERK activity by an NMDA receptor (NMDAR) antagonist decreased ERK activity and cocaine seeking on withdrawal day 30 [68]. Finally, local CeA injections of a metabotropic glutamate 2/3 (mGluR2/3) agonist that inhibits evoked glutamate transmission decreased cocaine seeking during late (21 d) but not early (3 d) withdrawal from cocaine [69].

There is also evidence for a role of CeA ERK activity in the incubation of morphine craving, as assessed in a conditioned place preference (CPP) procedure [70]. Time-dependent increases in morphine CPP after withdrawal (the operational measure of incubation of craving in the CPP procedure) correlate with increased phosphorylation of ERK and cAMP response element-binding (CREB; a transcription factor that is a downstream target of ERK) in CeA but not BLA. Inhibition of CeA but not BLA ERK activity decreased the enhanced morphine CPP after 14 withdrawal days, while stimulation of CeA ERK activity by NMDA enhanced drug CPP on withdrawal day 1, an effect that was reversed by pharmacological inhibition of ERK activation [70]. These findings indicate that incubation of morphine craving, as assessed in the CPP procedure, is mediated by activation of CeA ERK activity. A future research question will be to determine the exact role that CeA CREB plays in incubation of craving; however, this will require an experimental method that selectively inhibits CREB activity while leaving ERK activity intact.

Taken together, the studies described above indicate that glutamate-mediated ERK activation in the CeA is critical for the expression of incubation of cocaine and morphine craving. An important area for future research will be to determine the role that ERK signaling pathways in other brain areas play in the incubation of drug craving. As mentioned above, incubation of cocaine craving is associated with time-dependent increases in mPFC ERK activity. However, unlike the effects of CeA manipulations, pharmacological inhibition of ERK activity in either the dorsal or ventral mPFC on withdrawal day 30 had no effect on cocaine seeking [54], indicating that mPFC ERK activity does not contribute to incubation of cocaine craving. Time-dependent increases in cocaine seeking (as assessed in extinction tests) are also associated with increased ERK activity in NAc core [71]. However, it is unknown whether NAc ERK activity plays a causal role in incubation of cocaine craving, because the effect of inhibition of local ERK activity on cocaine seeking was not assessed. Finally, there is evidence implicating ERK activity in the VTA, downstream of GDNF and BDNF signaling, in the incubation of cocaine craving, which we discuss in the next section.

Finally, the cellular mechanisms downstream of CeA ERK activation that contribute to the incubation of craving are unknown. Because of the short timeframe for the behavioral tests (i.e., 30 min), Lu et al. [61] proposed that incubation of drug craving involves cue-triggered, ERK-mediated, rapidly-induced (within minutes) increases in neuronal excitability and synaptic transmission. These acute effects may involve ERK-mediated inactivation of voltage-gated potassium channels containing the Kv4.2 subunit, leading to decreased outward K^+ current, resulting in increased membrane depolarization [72], and consequently increased responsiveness of neurons to cue-induced neuronal activity. ERK's role in incubation of craving may also involve cue-induced ERK-mediated enhancement of excitatory neurotransmission by AMPAR insertion into cell membranes [73]. This hypothesis fits well with the data described above on the effect of inhibition of CeA glutamate transmission on incubation of cocaine craving.

Role of BDNF and GDNF in the VTA and NAc

BDNF and GDNF are neurotrophic factors that are important for the survival and function of midbrain dopamine neurons [74–76]. These neurotrophic factors also play a significant role in synaptic plasticity during development as well plasticity events associated with learning and memory [63, 77]. Results from many studies indicate that drugs of abuse alter BDNF signaling in the mesolimbic dopamine system and that BDNF activity promotes the behavioral effects of drugs [64, 66]. On the other hand, GDNF activity in the mesolimbic and nigrostriatal dopamine systems plays a complex role in the behavioral effects of abused drugs across drug classes, with evidence of both promoting and inhibiting drug-related behaviors [65–67, 78].

The first evidence for a role of BDNF in the incubation of cocaine craving was that time-dependent increases in cocaine seeking over the first 90 d of withdrawal correlate with time-dependent increases in BDNF protein levels in the VTA, NAc, and amygdala [20]. A subsequent study assessed whether VTA BDNF injections, given immediately after the last cocaine self-administration session, would enhance cocaine seeking in rats during early withdrawal [79], a time when cocaine seeking levels are low [4, 18]. Cocaine seeking on withdrawal days 3 and 10 was found to be enhanced in the BDNF-treated rats, as compared to vehicle-injected control rats, an effect that persisted for up to 30 days [79]. This effect of VTA BDNF injections was reversed by pharmacological inhibition of ERK activation [79]. The behavioral effects of BDNF injections do not appear to be due to its acute actions on synaptic transmission, because VTA BDNF injections 2 h before testing on withdrawal day 3 had no effect on cocaine seeking [79]. Thus, VTA BDNF injections likely affect long-term mechanisms underlying incubation of cocaine craving rather than having an immediate acute effect on drug seeking.

While these results [20, 79] suggest a role of mesolimbic BDNF in incubation of cocaine craving, they fall short of demonstrating a causal role of BDNF in this incubation, because the effect of interfering with endogenous BDNF function [80–82] was not assessed. Another issue is that the high local concentrations of exogenous BDNF after injections do not mimic the modest increases in endogenous BDNF in the VTA that are observed after withdrawal from cocaine [20]. An additional methodological issue in the BDNF injection study [79], and other studies described below on the effect of region-specific BDNF injections, is that BDNF is transported from cell bodies to axon terminals [83], and thus can change behavior by acting on unknown distal brain sites.

Another study demonstrated that daily NAc shell BDNF injections during cocaine self-administration training resulted in enhanced extinction responding and cue-induced reinstatement during tests performed after 11–15 withdrawal days [80]. Additionally, local delivery of an anti-BDNF antibody during self-administration training had the opposite effect, suggesting a causal role of endogenous BDNF in the NAc shell in enhancing cocaine seeking after withdrawal. However, this study did not assess the effect of exogenous BDNF or anti-BDNF antibody delivery on cocaine seeking during early withdrawal [80]. Thus, it is unknown whether NAc shell BDNF is critical for the incubation of cocaine craving or plays a more general role in cocaine seeking, independent of the withdrawal period. Finally, in contrast to the above studies, it was reported that dorsal mPFC BDNF injections, which increased BDNF levels in the NAc a day later, decreased cocaine seeking after 1 or 6 withdrawal days [84]. The relevance of these findings to the incubation of cocaine craving is currently unknown, because under the authors' training conditions (2-h daily sessions), cocaine seeking does not increase over time. Additionally, as discussed above, results on the effect of site-specific exogenous BDNF injections should be interpreted with caution.

With respect to GDNF, results from a recent study indicate an important role for this neurotrophic factor within the VTA in the incubation of cocaine craving [85]. VTA injections of an adeno-associated virus (AAV) vector containing rat GDNF cDNA on withdrawal day 1 increased cocaine seeking on days 11 and 31 [85]. Additionally, VTA but not SNA GDNF injections immediately after the last cocaine self-administration session increased drug seeking on withdrawal days 3 and 10; this effect of exogenous GDNF was reversed by local pharmacological inhibition of ERK activity. Importantly, interfering with VTA GDNF function by chronic delivery of anti-GDNF monoclonal neutralizing antibodies during withdrawal days 1–14 prevented the time-dependent increases in cocaine seeking on withdrawal days 11 and 31 [85]. These results suggest that during the first weeks of withdrawal from cocaine, GDNF-dependent neuroadaptations in VTA are critical for the development of incubation of cocaine craving.

The role of VTA and NAc GDNF in the incubation of craving to heroin was studied in a subsequent experiment, to address the question of whether this cellular mechanism was specific to cocaine or more broadly relevant to other drugs of abuse [86]. Time-dependent increases in heroin seeking were associated with time-dependent changes in GDNF mRNA expression in VTA and NAc during the first month of withdrawal from heroin. Additionally, NAc, but not VTA, GDNF injections, given immediately after the last drug self-administration session, enhanced heroin seeking after withdrawal. However, subsequent results indicate that, unlike for cocaine, endogenous GDNF in VTA or NAc does not contribute to incubation of heroin craving. Specifically, GDNF protein levels were not altered in the VTA or NAc after 1, 11, or 30 withdrawal days, and time-dependent increases in heroin seeking after withdrawal were not affected by VTA or NAc delivery of anti-GDNF neutralizing antibodies [86].

In summary, incubation of cocaine craving is associated with time-dependent increases in BDNF expression in mesolimbic areas, and VTA or NAc shell BDNF injections cause delayed and long-lasting increases in cocaine seeking after withdrawal. The data on the effect of interfering with local BDNF function in NAc shell on cocaine seeking [80], together with the BDNF expression data [20], suggest that NAc BDNF plays a role in incubation of cocaine craving. This putative role, however, should be confirmed in future studies assessing the effect of interfering with NAc BDNF signaling on the development and expression of the incubation of cocaine craving. There is also evidence that endogenous GDNF activity in the VTA is critical for the development of incubation of cocaine (but not heroin) craving. Finally, the findings that inhibition of ERK activity in VTA reverses the effect of exogenous administration of both BDNF and GDNF in VTA on incubation of cocaine craving suggest that local ERK activity contributes to the effects of these growth factors on incubation of cocaine craving (see Fig. 3).

Roles for GluA2-lacking AMPARs in the NAc

AMPARs are homo- or hetero-tetrameric glutamate-gated ion channels. GluA2-lacking AMPARs are comprised of GluA1, GluA3, or GluA4, but not GluA2, subunits. They differ from GluA2-containing AMPARs in several physiological characteristics, including greater channel conductance, increased calcium permeability, and inwardly rectified currents due to voltage-dependent block by polyamines [87, 88] (Fig. 4). Because of these cellular characteristics, GluA2-lacking AMPARs are more susceptible to neuronal stimulation than GluA2-containing AMPARs [87, 88]. Results from several recent studies indicate that prolonged withdrawal from cocaine self-administration results in upregulated synaptic expression of GluA2-lacking AMPARs in the NAc (reviewed in [89]).

In a recent study, *in vivo* surface and intracellular expression of NAc GluA1, GluA2, and GluA3 AMPAR subunits were quantified in rats trained to self-administer cocaine (or

saline) and tested for cocaine seeking after 1 or 45 withdrawal days [90]. Levels of surface, intracellular, and total GluA1-containing AMPARs increased from 1 day to 45 withdrawal days; in contrast, no time-dependent alterations of GluA2 and GluA3-containing AMPARs were observed. These results suggest that after prolonged withdrawal from cocaine, the normal complement of NAc GluA2-containing AMPARs is supplemented by the addition of GluA2-lacking AMPARs (GluA1/3 and/or homomeric GluA1).

The insertion of GluA2-lacking AMPARs at NAc glutamatergic synapses was confirmed by whole-cell patch clamp electrophysiological recordings [90]. Current-voltage assessment of evoked excitatory postsynaptic currents (EPSCs) in NAc output neurons revealed greater inward rectification in cocaine-exposed versus saline-exposed rats. Bath application of a selective blocker of GluA2-lacking AMPARs reduced evoked EPSC amplitude only in neurons recorded from the cocaine-exposed but not saline-exposed rats. Most importantly, selective pharmacological blockade of NAc GluA2-lacking AMPARs decreased cocaine seeking after 45 withdrawal days but not 1 day. Taken together, these data demonstrated a causal role of these newly formed GluA2-lacking AMPARs in incubation of cocaine craving [90].

The formation of NAc GluA2-lacking AMPARs was recently confirmed in mice and rats trained to self-administer cocaine and tested for cocaine seeking after 35–49 withdrawal days [91–93]. However, these results, and those described above, are not consistent with results from earlier studies in which it was found that incubation of cocaine craving after withdrawal is potentiated in GluA1-knockout mice [21] or that viral over-expression of GluA1 or GluA2 AMPAR subunits in the NAc shell decrease extinction responding during early withdrawal from cocaine [94]. The results from these earlier studies, however, should be interpreted with caution. Many compensatory changes likely occur in the brains of GluA1-knockout mice [21], because of the absence of the AMPAR subunit during development; these compensatory changes can alter cocaine seeking independent of the normal role of GluA1 subunit in behavior. In the case of the viral over-expression study [94] the viral vector that was utilized consisted of a powerful promoter that increased GluA1 or GluA2 AMPARs expression well beyond the normal or drug-induced physiological range.

In summary, the studies reviewed above indicate that prolonged, but not short, withdrawal from cocaine self-administration causes the formation of GluA2-lacking AMPARs in the NAc. The synaptic incorporation of these newly formed receptors can enhance the responsiveness of NAc neurons to glutamatergic inputs from cortical and limbic regions, due to both the increases in the absolute number of surface AMPARs and the higher conductance of GluA2-lacking AMPARs [89]. Consequently, when cocaine-associated cues are presented after prolonged drug withdrawal, and glutamate is released in the NAc [95, 96], local NAc neurons would respond more robustly, leading to enhanced cocaine seeking (i.e., an incubated response) [90].

Finally, the mechanisms underlying the delayed formation of NAc GluA2-lacking AMPARs after withdrawal from cocaine are largely unknown. It has been reported that genetic deletion of NMDARs in VTA dopamine neurons of adult mice, which decreases cocaine-induced VTA synaptic plasticity during early withdrawal [97], prevented the formation of NAc GluA2-lacking AMPARs and decreased cocaine seeking after 35 withdrawal days [91]. This suggests that early cocaine-induced plasticity in VTA plays a role in the formation of NAc GluA2-lacking AMPARs via a mechanism that has yet to be determined. Another potential mechanism for the formation of GluA2-lacking AMPARs after prolonged withdrawal might involve the progressive increase of BDNF levels in NAc [20]. *In vitro* studies indicate that BDNF application can cause synaptic insertion of GluA2-lacking AMPARs (for a review see [98]). Additionally, the time course of the formation of NAc

GluA2-lacking AMPARs after withdrawal [89] resembles the time course of increases in NAc BDNF levels [20], and preliminary experiments suggest that acute NAc BDNF injections are able to increase the surface expression of GluA2-lacking AMPARs [Li X, Dejoseph, MR, Bahi A, Dreyer J-L, Urban JH, Wolf ME (2010) Brain-derived neurotrophic factor (BDNF) and cocaine-induced AMPA receptor plasticity in the rat nucleus accumbens. Society for Neuroscience Abstracts, 366.16].

Concluding remarks

In this review, we discussed recent findings which have advanced our understanding of neural mechanisms underlying the incubation of drug craving. These studies indicate that signaling pathways in the VTA that are downstream of the neurotrophins BDNF and GDNF play an important role in the incubation of cocaine craving. There is also evidence that neuronal activation of the ventral mPFC and ERK activation in the CeA mediate this incubation. Finally, results from several recent studies indicate that the formation of GluA2-lacking AMPARs in the NAc plays a critical role in the incubation of cocaine craving (Fig. 2). Below, we briefly discuss incubation of cocaine craving from a circuit perspective.

Incubation of cocaine craving is mediated by increased neuronal activity in the ventral mPFC, NAc core, and CeA [16, 54, 68]. However, while there are excitatory projections from the ventral mPFC to CeA [99], electrophysiological studies show that ventral mPFC stimulation decreases CeA neuronal activity [100]. Additionally, output neurons from the CeA do not directly project to the NAc core. Furthermore, ventral mPFC neurons primarily project to the NAc shell rather than the core [101], and the dorsal mPFC, which primarily projects to the NAc core [101], does not appear to contribute to the incubation of cocaine craving. This “circuit incompatibility” suggests that several cortico-striatal circuits likely mediate incubation of cocaine craving. This is a likely possibility, because the main operational measure of the incubation of drug craving, operant responding in extinction tests, involves exposing rats to both contextual and discrete cues previously paired with drug reward. Thus, incubation of drug craving likely involves time-dependent increases in responding to both cue types, whose neurobiological substrates only partially overlap [102–106]. It is clear that while much has been discovered about the neurobiology of drug craving since it was first described over two decades ago, many unanswered questions remain (Box 1).

Finally, it is beyond the scope of this review to discuss clinical implications of incubation of drug craving, a topic that has been discussed previously (for reviews see [40, 107]). It is our hope that the recent demonstration of incubation of tobacco craving in human smokers [108] will encourage investigators to study brain mechanisms and genetic factors of incubation of drug craving in humans.

Box 1: Outstanding questions and future directions

1. Are there similarities in the mechanisms of incubation across drug and non-drug rewards? Currently there is evidence for both similarities and differences. Glutamate-mediated ERK activation in the CeA is critical for the incubation of cocaine and morphine craving [68, 70], and inhibition of glutamate transmission in the CeA decreases both cocaine and sucrose craving [69, 109]. In contrast, GDNF signaling in the VTA, which is critical for the incubation of cocaine craving [85], does not play a role in the incubation of heroin craving [86].
2. Are there similarities in the mechanisms underlying incubation of the conditioned response to appetitive versus aversive cues after behavioral conditioning? Time-dependent increases in conditioned responses also occur

under certain experimental conditions when neutral cues are repeatedly paired with aversive events like footshock, a phenomenon termed incubation of fear [110–112]. As the expression of conditioned fear is critically dependent on CeA activity [113], an interesting question for future research is whether glutamatergic transmission in CeA, which mediates incubation of cocaine [68, 69], morphine [70], and sucrose [109] craving, also mediates incubation of fear.

3. Does the incubation of cocaine craving involve a suppression of cue responding during early withdrawal? Studies on incubation of craving reviewed here have been primarily guided by the hypothesis that some drug-induced brain neuroadaptations progressively increase after withdrawal, leading to enhanced responding to drug cues [40]. However, studies in which the training session duration and the number of training days were manipulated [17, 56] indicate that extended cocaine exposure does not *increase* responding to cocaine cues during late withdrawal but rather *decreases* responding to these cues during early withdrawal. These behavioral data suggest that incubation of craving also involves drug-induced neuroadaptations that suppress cue responding during early withdrawal, and that these suppressor mechanisms dissipate over time [43, 114, 115]. The identification of these *suppressive* mechanisms is a promising future research direction.

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Glossary

ACQUISITION OF A NEW RESPONSE	A procedure in which an animal learns to make a new operant response (e.g., a lever press) reinforced solely by a cue previously paired with a reward (e.g., drug) earned by performing a different operant response (e.g., a nose poke).
CONDITIONED PLACE PREFERENCE (CPP)	A classical (Pavlovian) conditioning procedure in which one distinct context is paired with drug injections while another context is paired with vehicle injections. During a subsequent drug-free test, the animal chooses between the drug- and the vehicle-paired contexts. An increase in preference for the drug context serves as a measure of the drug's Pavlovian rewarding effects.
CUE-INDUCED REINSTATEMENT	A procedure in which rats are trained to self-administer a drug; each reward delivery is temporally paired with a discrete cue (e.g. tone and light). Lever pressing is then extinguished in the absence of the discrete cue. During reinstatement testing, exposure to the discrete cue, which is earned contingently by responding on the drug-associated lever, reinstates drug seeking.
DRUG CRAVING	Refers to an affective state that can be induced in human drug users by exposure to the drug itself, drug-associated cues, or stress. In laboratory animals, craving is often inferred from the subjects' behavioral response (e.g., lever-

	pressing) to drugs, drug-associated cues, or stress. The inference of an affective state from observed behavioral responses is not unique to drug addiction. For example, subjective states of fear or hunger are often inferred from observing freezing or feeding behavior, respectively, in rodents and other species.
DRUG PRIMING	In reinstatement studies, drug priming refers to non-contingent injections of the self-administered drug or other drugs just prior to the reinstatement tests under extinction conditions.
EXTINCTION	A decrease in the frequency or intensity of learned responses after the removal of the unconditioned stimulus (e.g. food, drug) that has reinforced the learning. In drug self-administration studies, extinction of lever responding is typically performed in the presence of contextual cues previously associated with drug availability (i.e., houselight, lever). In CPP studies, extinction typically involves exposing rodents to the previously drug-paired context in the drug-free state.
INCUBATION OF DRUG CRAVING	A hypothetical motivational process inferred from the findings of time-dependent increases in cue-induced drug seeking after withdrawal from drug self-administration in rats [4] and time-dependent increases in cue-induced subjective self-reports of craving during abstinence in humans [108].
PSYCHOMOTOR SENSITIZATION	A progressive increase in locomotor activity or stereotypy with repeated drug (e.g., cocaine) administration.
RECONSOLIDATION (OF MEMORY)	a hypothetical memory process in which a consolidated memory item becomes labile upon its retrieval and undergoes a protein-dependent re-stabilization process.
REINSTATEMENT	In studies of reinstatement of drug seeking, reinstatement refers to the resumption of drug seeking after extinction following exposure to drugs, drug cues, or stressors.
RELAPSE	A term used to describe the resumption of drug-taking behavior during self-imposed or forced abstinence in humans.

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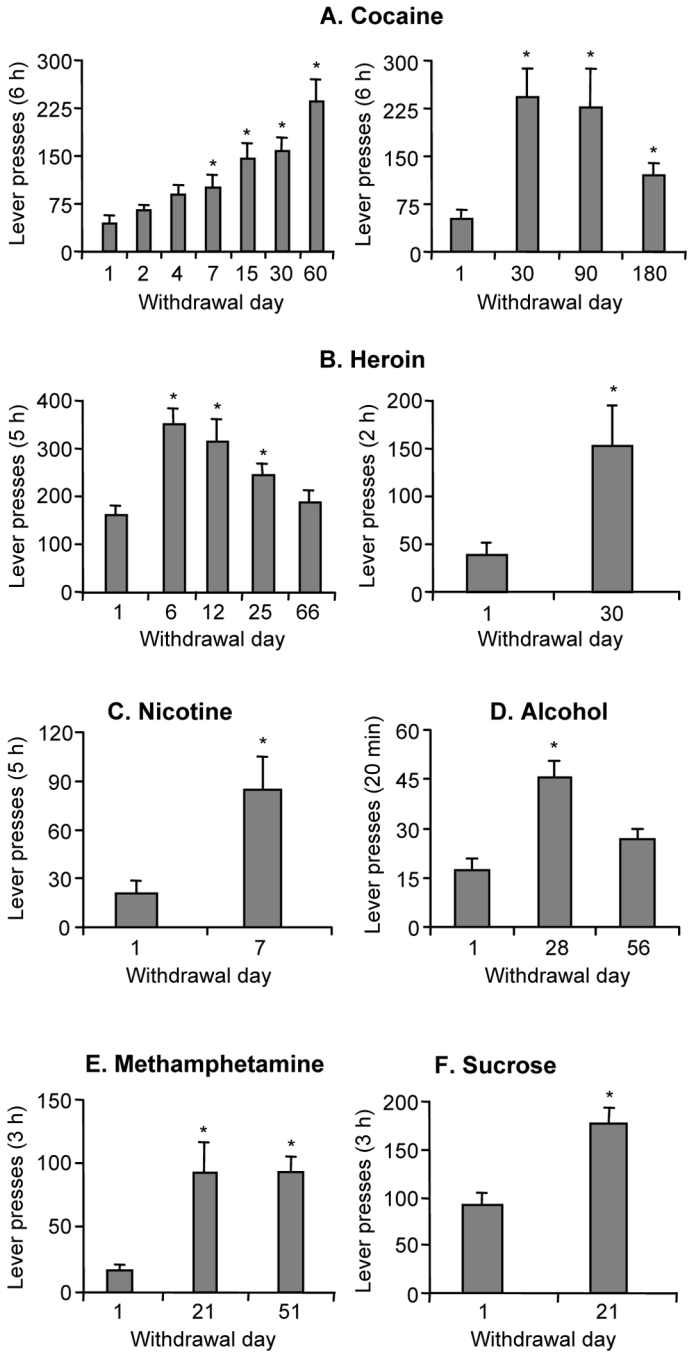

Figure 1. Incubation of reward craving

Extinction tests: Data are mean (±SEM) number of non-reinforced responses on a lever previously paired with reward delivery during self-administration at different days after withdrawal from (A) cocaine (left panel, [4]; right panel, [18]), (B) heroin [left panel, [6], right panel, [86]), (C) nicotine [9], (D) alcohol [8], (E) methamphetamine [7], or (F) oral sucrose [109]. During the extinction tests in [6, 7, 18, 86, 109], rats were re-exposed to the reward-associated environment (i.e., the self-administration chambers) and lever presses led to contingent presentations of a discrete cue previously paired with reward delivery. During the extinction tests in [4, 8, 9], rats were re-exposed to the self-administration chambers and

lever presses had no reinforced consequences. * Different from withdrawal day 1, $p<0.05$. Data were redrawn, with permission, from the above cited references.

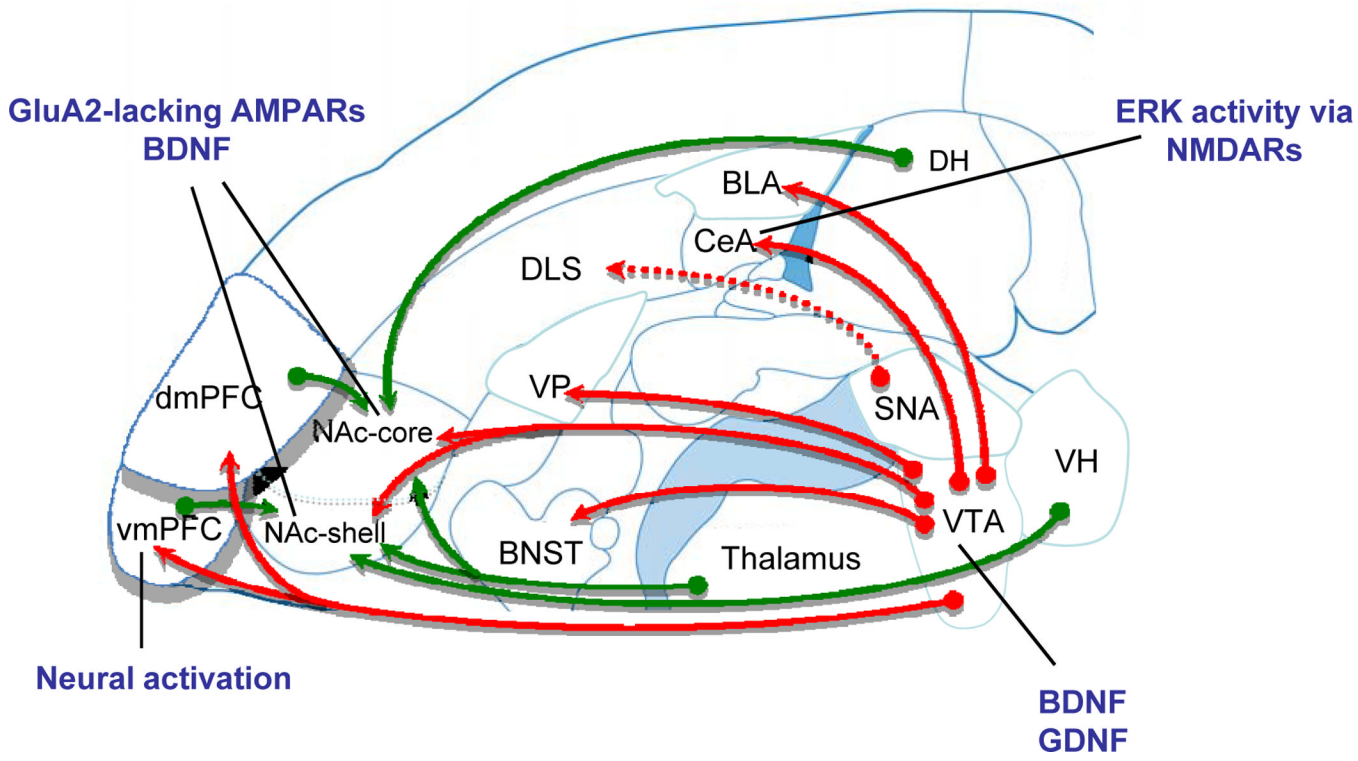

Figure 2. Brain regions and molecular mechanisms involved in incubation of cocaine craving

Horizontal section of a rat brain [116] depicting the mesocorticolimbic (red arrows) and nigrostriatal (red dashed arrows) dopamine systems and the glutamatergic projections (green arrows) to the NAc. The depicted mechanisms, which are summarized in detail in the text, refer to the roles in incubation of cocaine craving of ventral mPFC (vmPFC) neuronal activity [54], GluA2-lacking AMPARs in the NAc [89–91], BDNF signaling in the NAc and VTA [20, 79, 80], GDNF activity in the VTA [85], and CeA ERK activity and glutamate transmission [68, 69]. Abbreviations: BLA and CeA, basolateral and central nuclei of the amygdala; BNST, bed nucleus of the stria terminalis: DH, dorsal hippocampus; DLS, dorsolateral striatum; dmPFC and vmPFC, dorsal and ventral medial prefrontal cortex; SNA, substantia nigra; VH, ventral hippocampus; VP, ventral pallidum.

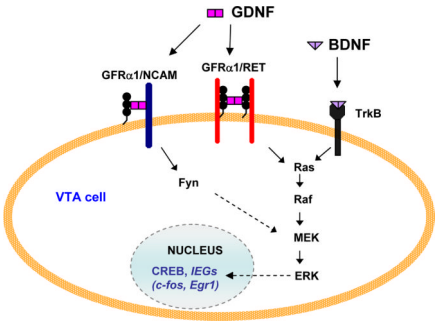

Figure 3. A putative signaling cascade involving the activation of ERK through GDNF and BDNF signaling in the VTA and its importance to the incubation of cocaine craving

Increased secreted GDNF can increase ERK activity via its actions on RET tyrosine kinase/glial cell line-derived receptor alpha 1 (GFRα1) [117] or GFRα1/neural cell adhesion molecule (NCAM) [118] signaling mechanisms. Increased extracellular BDNF can increase ERK activity via its actions on the TrkB neurotrophin receptor [63]. Increased ERK phosphorylation can activate transcription factors like CREB and immediate early genes (IEG) like c-fos and Egr1 that play a role in BDNF- and GDNF-induced long-term neuroadaptations that potentially contribute to the incubation of cocaine craving.

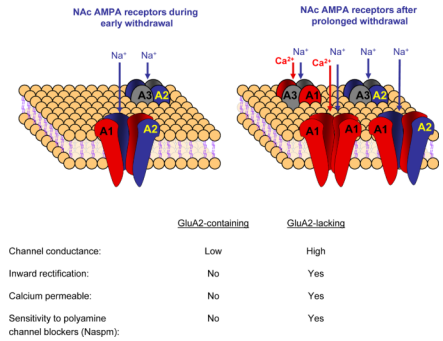

Figure 4. Physiological differences between GluA2-lacking and GluA2-containing AMPARs in the brain and their relevance for incubation of cocaine craving

GluA2-lacking AMPARs differ from GluA2-containing AMPARs in several physiological characteristics, including channel conductance, permeability to calcium ions, and inward rectification [87, 88]. The formation of GluA2-lacking AMPARs has been observed in the NAc after prolonged withdrawal from cocaine (reviewed in [89]). The synaptic incorporation of these new receptors can enhance the responsiveness of NAc neurons to glutamatergic inputs from cortical and limbic regions, due to both the increases in the absolute number of surface AMPARs and the higher conductance of GluA2-lacking AMPARs [90]. As discussed in the text, there is evidence that these newly formed GluA2-lacking AMPARs in the NAc play a critical role in the incubation of cocaine craving.