REGULATION OF NETRIN-1 RECEPTORS BY AMPHETAMINE IN THE ADULT BRAIN

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Abstract

Netrin-1 is a guidance cue molecule fundamental to the organization of neuronal connectivity during development. Netrin-1 and its receptors, deleted in colorectal cancer (DCC) and UNC-5 homologues (UNC-5), continue to be expressed in the adult brain, although neither their function nor the kinds of events that activate their expression are known. Two lines of evidence suggest a role for netrin-1 in amphetamine-induced dopamine plasticity in the adult. First, DCC is highly expressed by adult dopamine neurons. Second, adult mice with reduced DCC levels do not develop amphetamine-induced behavioral sensitization. To explore the role of netrin-1 in amphetamine-induced plasticity, we examined the effects of sensitizing treatment regimens of amphetamine on DCC and/or UNC-5 protein expression in the adult rat. These treatments produced striking and enduring increases in DCC and UNC-5 expression in the cell body, but not terminal regions, of the mesocorticolimbic dopamine system. Notably, neuroadaptations in the cell body region of mesocorticolimbic dopamine neurons underlie the development of sensitization to the effects of amphetamine. Furthermore, these localized amphetamine-induced changes were prevented by co-treatment with an *N*-methyl-D-aspartate receptor antagonist, a treatment known to block the development of amphetamine-induced sensitization of behavioral activation, dopamine release and motivated behavior. Using immunohistochemistry, we showed that both DCC and UNC-5 receptors are highly expressed by adult mesocorticolimbic dopamine neurons. These results provide the first evidence that repeated exposure to a stimulant drug such as amphetamine affects netrin-1 receptor expression in the adult brain. Taken together, our findings suggest that changes in netrin-1 receptor expression may play a role in the lasting effects of exposure to amphetamine and other stimulant drugs.

Keywords

sensitization; DCC; UNC-5; plasticity; dopamine; glutamate

Stimulant drugs, such as amphetamine, induce locomotor-activating and rewarding effects by increasing extracellular dopamine (DA) levels in striatal terminal regions. These behavioral and neurochemical effects become sensitized when the drug is administered repeatedly (Stewart and Badiani, 1993; Kalivas and Stewart, 1991; Vezina 2004). Key features of sensitization, including its gradual development and persistence over time,

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suggest underlying alterations in the organization of mesocorticolimbic DA circuitry. Indeed, stimulant-induced modifications in dendritic structure have been identified in midbrain DA perikarya and in striatal and cortical DA terminal regions, suggesting alterations in patterns of synaptic connectivity within these regions (Berlanga et al., 2006; Kolb et al., 2003; Li et al., 2003; Mueller et al., 2006; Robinson and Kolb, 1997, 1999; Sarti et al., 2007). Although the mechanisms underlying amphetamine-induced plasticity of DA circuitry are unclear, it is known that the development of sensitization depends on (1) the direct actions of amphetamine in the ventral tegmental area (VTA), the cell body region of mesocorticolimbic DA neurons (Bjijou et al., 1996; Cador et al., 1995; Hooks et al., 1992; Kalivas and Weber, 1988; Vezina, 1993, 1996, 2004; Vezina and Stewart, 1990), and (2) amphetamine-induced glutamatergic transmission within this region (Cador et al., 1999; Wolf, 1998; Wolf and Xue, 1998, 1999; Wolf et al., 2000; Xue et al., 1996). How these initial events at the cell body region translate into the development of sensitization and a reorganization of mesocorticolimbic DA circuitry is poorly understood.

A potential candidate for mediating amphetamine-induced reorganization of DA circuitry is the netrin family of guidance cues; proteins known for their role in directing the outgrowth of extending axons and dendrites to their appropriate targets (Furrer et al., 2003; Manitt and Kennedy, 2002; Suli et al., 2006). Netrin-1 is a bifunctional cue that participates in the organization of brain circuitry by stimulating neurite attraction through DCC (deleted in colorectal cancer) receptors or repulsion through DCC/UNC-5 homologues (UNC-5) receptor complexes (Bartoe et al., 2006; Bouchard et al., 2004; Hong et al., 1999; Manitt and Kennedy, 2002; Stein et al., 2001; Williams et al., 2003). Therefore, the balance between DCC and UNC-5 receptor expression should determine the effect that netrin-1 will have on a particular neuron at a specific point in time.

Interestingly, netrin-1 and its receptors are not only expressed during development, but continue to be expressed in the adult CNS (Livesey and Hunt, 1997; Manitt et al., 2001, 2004; Manitt et al., 2006; Volenec et al., 1997, 1998). While the function of netrin-1 in adulthood is unclear, there are two lines of evidence that suggest a potential role for netrin-1 in amphetamine-induced plasticity of the adult DA system. First, DCC is highly expressed by DA neurons in the adult brain (Osborne et al., 2005). Second, we have recently shown that adult mice with reduced levels of DCC do not develop sensitization to the behavioral effects of amphetamine when treated repeatedly (Flores et al., 2005).

To begin to explore the role of netrin-1 signaling in amphetamine-induced plasticity, we examined if sensitizing regimens of amphetamine regulate the expression of DCC and/or UNC-5 receptors at DA cell body and/or terminal regions in the adult rat. In addition, we investigated if, similar to other drug-induced neuroadaptations, the regulation of netrin-1 receptors by amphetamine depends on *N*-methyl-D-aspartate (NMDA) glutamatergic neurotransmission. We also assessed whether VTA DA neurons express netrin-1 receptors in the adult rat using double-labeling immunofluorescence. Although it has previously been shown that DCC receptors are expressed by adult midbrain DA neurons (Osborne et al., 2005), the pattern of UNC-5 expression remains unknown.

EXPERIMENTAL PROCEDURES

Subjects

Adult male Sprague–Dawley rats (Charles River Canada, St. Constant, Quebec, Canada) weighing 250 g at the start of all experiments were used. Animals were housed in pairs under a 12-h light/dark cycle. Food and water were available *ad libitum*. Experimental procedures were conducted during the light cycle. All experiments were performed in accordance with the guidelines of the Canadian Council of Animal Care and conformed to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the McGill University/Douglas Hospital Animal Care Committee and all efforts were made to minimize pain and suffering, and to reduce the number of animals used.

Drugs

d-Amphetamine sulfate (AMPH; Sigma-Aldrich, Dorset, UK) was dissolved in 0.9% saline. The selective NMDA receptor antagonist, 3-(2-carboxypiperazine-4-yl) propyl-1-phosphonic acid (CPP; Sigma-Aldrich, Milwaukee, MI, USA) was also prepared by dissolution in 0.9% saline. All injections were administered i.p.

Apparatus

The locomotor boxes (AccuScan Instruments, Inc., Columbus, OH, USA) used in these experiments were made of acrylic and activity was monitored by sensors located on the sides, front, and back of the boxes. Data collection was performed online using the Versamax Software (version 4.0, 2004); AccuScan Instruments, Inc., Columbus, OH, USA.

Behavioral experiments

Experiment 1—Prior to the first pretreatment day, all animals were habituated to the testing environment for 30 min and locomotor activity was recorded. Animals were then matched on their locomotor scores and assigned to AMPH ($n=7$) or saline ($n=7$) pretreatment groups. On days 1–4 (Fig. 1a), animals were treated with i.p. injections of AMPH (3 mg/kg) or 0.9% saline. Locomotor activity was recorded for 90 min following each injection. On day 12 (i.e. one week following the last pretreatment day) a test for behavioral sensitization was conducted. To this end, all animals, regardless of pretreatment group, were administered a challenge dose of AMPH (1 mg/kg, i.p.) and their locomotor activity was recorded for 90 min.

Locomotor activity is defined as the total distance traveled, in cm, by the animal in a given period of time. The dose of AMPH given during the pretreatment phase is one that increases locomotion and, after repeated administration, stereotypy. For the test phase, the dose was reduced to reveal primarily locomotion. Immediately following the test for sensitization, all animals were killed by decapitation and their brains were processed for Western blot analysis.

Experiment 2—The exact same procedures were used for the pretreatment phase as in experiment 1, but on the test day both AMPH ($n=8$) and saline ($n=6$) pretreatment groups

were administered an i.p. injection of saline, and locomotor activity was recorded for 90 min (Fig. 1b). All animals were then killed by decapitation and their brains processed for Western blot analysis. Importantly, only those proteins whose expression was found to be altered by the AMPH treatment in experiment 1 were examined.

Experiment 3—Similar procedures were used for the pre-treatment phase as in experiment 1 (Fig. 1c), except that, during the pretreatment phase, animals received i.p. injections of the selective NMDA receptor antagonist, CPP (4 mg/kg), or saline in the animal colony 30 min prior to the i.p. injections of AMPH or saline administered in the activity room. The dose of CPP was previously shown to effectively block the development of sensitization to AMPH (Flores et al., 2000). The test for behavioral sensitization was conducted on day 12, and all four groups, saline-AMPH ($n=12$); saline-saline ($n=11$); CPP-AMPH ($n=7$); and CPP-saline ($n=7$) were administered a challenge dose of AMPH (1 mg/kg, i.p.) only. Immediately following the test for sensitization, all animals were killed by decapitation and their brains were processed for Western blot analysis.

Western blotting

The brains obtained from experiments 1–3 were flash-frozen in 2-methyl-butane. Bilateral punches of the VTA, nucleus accumbens (NAcc), including core and shell, and medial prefrontal cortex (mPFC), including cingulate areas 1 and 2, were taken from 1 mm-thick coronal sections. Western blotting was conducted as described previously (Flores et al., 2005). Briefly, protein samples (25 μ g) were resolved using 7.5% SDS-PAGE and transferred to nitrocellulose membranes which were then incubated overnight at 4 °C with primary antibodies against DCC (G97-449; 1:1000; mouse monoclonal, Pharmingen, Mississauga, Ontario, Canada), UNC-5 (pan-UNC-5 antiserum, 1:7500, kindly provided by Dr. Tony Pawson, University of Toronto), and tubulin (1:4000; mouse monoclonal, Sigma-Aldrich). Importantly, all proteins for each brain region were assessed using the same membrane, with tubulin used as a control for any errors in loading. Primary antibody incubation was followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Vector Scientific, Torrance, CA, USA). Protein expression was detected using chemiluminescence (Perkin Elmer, Waltham, MA, USA), and signal intensity was quantified on scanned images of the immunoblots (Kodak 1D Image Analysis Software, 2000, New Haven, CT, USA).

Immunohistochemistry

Perfusion and histology—Rats were anesthetized with an overdose of sodium pentobarbital (>65 mg/kg i.p.) and perfused intracardially with 250 ml 0.9% saline followed by 400 ml of fixative solution (4% paraformaldehyde and 0.2% picric acid in 0.1 M phosphate buffer). Following perfusion, brains were post-fixed for 50 min at 4 °C and cryoprotected (30% sucrose in phosphate-buffered saline; PBS) overnight at 4 °C. Brains were then flash-frozen in 2-methyl-butane and immediately sectioned using a Leica SM2000-R sliding microtome.

Immunofluorescence—Free floating sections (40 μ m) were processed for dual-labeling immunofluorescence. Briefly, sections were collected and rinsed in PBS and incubated in

blocking solution (2% bovine serum albumin, 0.2% Tween-20 in PBS) for 1 h at RT. Sections were then incubated overnight at 4 °C in primary antibodies against DCC (1:500) and tyrosine hydroxylase (TH; 1:500; rabbit polyclonal, Chemicon, Temecula, CA, USA) or in primary antibodies against pan-UNC-5 (1:5000) and TH (1:300; mouse monoclonal, Chemicon). Importantly, the antibodies against DCC and UNC-5 that were used for immunofluorescence were the same as those used for Western blot analyses. Sections were then washed several times in blocking solution and incubated with Alexa 488- and Alexa 546-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA) for 45 min at RT. As a negative control, adjacent sections were processed as described above except that they were incubated overnight at 4 °C in blocking solution without primary antibodies. The dilutions used in this study for DCC and UNC-5 antibodies were determined by testing different dilutions to obtain the optimal dilution for observing bright DCC or UNC-5 immunoreactivity and low background staining in midbrain sections. Immunofluorescence was visualized using a Leica DM4000B microscope and images were captured with a Microfire camera and PictureFrame software (Microbrightfield, Williston, VT, USA).

Statistical analyses

Behavioral sensitization was quantified as the difference in locomotor activity between AMPH- and saline-pretreated animals in response to an AMPH challenge. Separate two-way repeated measures analysis of variance (ANOVA) of pretreatment (drug and saline)×time was used to analyze group differences in experiments 1 and 3.

DCC and UNC-5 expression in each blot was standardized using tubulin. Immunoblots for each protein, within each brain region and for each experiment, were developed separately. Thus, differences in DCC and UNC-5 expression between AMPH- and saline-pretreated groups were analyzed on the raw data (i.e. actual signal intensity values) using independent samples Student *t*-tests. *t*-Tests were carried out only where differences were visually apparent. Data in the figures are expressed as percentages of the saline group. The criterion for significance was set at *P* 0.05.

RESULTS

Netrin-1 receptors are expressed by DA neurons

As a first step in examining the possible involvement of netrin-1 receptors in AMPH-induced plasticity of the mesocorticolimbic DA system, we performed co-immunofluorescence experiments to determine if mesocorticolimbic DA neurons express DCC and UNC-5 receptors. Qualitative analysis of midbrain sections revealed that the highest expression of these receptors is localized to DA cell body regions (Fig. 2). Furthermore, double-labeling experiments revealed that all but a few DCC-positive cells were TH-immunoreactive. Similarly, the majority of the UNC-5 positive cells observed in this region were TH-positive. DCC and UNC-5 co-immunostaining was also observed in TH-positive terminals within the mPFC and NAcc DA terminal regions (data not shown). These results are in agreement with those reported previously on DCC expression (Osborne et al., 2005) and are the first demonstration of UNC-5 expression in adult rat midbrain DA neurons.

AMPH upregulates netrin-1 receptors in the VTA

Experiment 1—The aim of this experiment was to examine if a sensitizing regimen of AMPH can regulate the expression of DCC and/or UNC-5 receptors in mesocorticolimbic DA cell body and/or terminal regions. Across all pretreatment days, AMPH-pretreated animals exhibited higher locomotor activity compared with saline-pretreated animals (data not shown).

Behavioral sensitization—Fig. 3a illustrates the mean locomotor activity counts obtained on the test for sensitization in AMPH- and saline-pretreated animals. AMPH-pretreated animals showed robust behavioral sensitization as they exhibited significantly higher activity levels in response to the AMPH challenge compared with saline-pretreated animals, which received the drug for the first time ($F_{(1, 12)} = 12.47$, $P < 0.01$).

Netrin-1 receptor protein expression—As described previously, the DCC antibody detected a single ~180 kDa band (Flores et al., 2005). The pan-UNC-5 antiserum, which has been shown to recognize three mammalian UNC-5 homologues (UNC-5H1, UNC-5H2, and UNC-5H3 (Manitt et al., 2004)) detected a single band of ~130 kDa.

As shown in Fig. 3b, there was a sizeable and significant increase in DCC (~150%; $t_{(12)} = 1.87$, $P < 0.04$) and UNC-5 (~100%, $t_{(11)} = 2.92$, $P < 0.01$) expression in the VTA of AMPH-pretreated animals, as compared with their saline-pretreated counterparts. No detectable difference in VTA tubulin expression was observed.

In the NAcc, no difference in DCC or UNC-5 expression was observed between AMPH- and saline-pretreated animals (Fig. 3c). In contrast, in the mPFC, AMPH-pre-treated animals exhibited greater UNC-5 expression compared with saline-pretreated animals (~140%, $t_{(10)} = 2.98$, $P < 0.01$). No difference in DCC or tubulin expression in the mPFC was detected between the two groups (Fig. 3d).

Experiment 2—This experiment was conducted to ensure that the effects observed on protein expression in experiment 1 were indeed a result of the AMPH pretreatment, and not of the AMPH challenge given on the test day. Across all pretreatment days, locomotor activity was significantly higher in the AMPH-pretreated group than in the saline-pretreated group. Importantly, on the test day, both groups were administered a saline challenge injection (data not shown).

Netrin-1 receptor protein expression—Similar to the results obtained in experiment 1, AMPH-pretreated animals given a saline challenge on the test day exhibited significant increases in the expression of DCC (~110%; $t_{(11)} = 2.81$, $P < 0.01$) and UNC-5 (~70%, $t_{(12)} = 1.89$, $P < 0.04$) in the VTA, compared with their saline-pretreated counterparts (Fig. 4a). In this experiment, however, the difference previously found in mPFC UNC-5 expression between AMPH- and saline-pretreated animals was no longer observed (Fig. 4b).

AMPH-induced upregulation of netrin-1 receptors requires NMDA receptor activation

Because glutamatergic neurotransmission is known to play a role in the development of sensitization to AMPH and to mediate many AMPH-induced neuroadaptations (Wolf, 1998),

we examined if administration of the NMDA receptor antagonist, CPP, would prevent the effects of AMPH on netrin-1 receptor expression observed in experiments 1 and 2. As previously shown, (Wolf, 1998; Flores et al., 2000), during the pretreatment phase, locomotor activity was significantly higher in the saline-AMPH- and CPP-AMPH-pretreated groups than in the saline-saline- and CPP-saline-pretreated groups (data not shown).

Behavioral sensitization—Consistent with previous findings (Flores et al., 2000), systemic administration of CPP during the pretreatment phase blocked the development of behavioral sensitization. On the sensitization test day, saline-AMPH-pretreated animals displayed behavioral sensitization as they exhibited significantly higher AMPH-induced activity levels compared with saline-saline-pretreated animals ($F_{(1, 21)} = 18.12$, $P < 0.01$; Fig. 5a). In contrast, CPP-AMPH-pretreated animals exhibited similar activity scores in response to the AMPH challenge as those that received the drug for the first time (i.e. CPP-saline-pretreated animals; $F_{(1, 12)} = .00001$, $P < 0.05$; Fig. 5b).

Netrin-1 receptor protein expression—As in experiments 1 and 2, significant increases in DCC (~60%, $t_{(12)} = 2.23$, $P < 0.01$) and UNC-5 (~160%, $t_{(12)} = 2.77$, $P < 0.01$) expression were detected in the VTA of saline-AMPH-pretreated animals when compared with saline-saline-pretreated animals (Fig. 5c, d). Remarkably, this increase was completely blocked by CPP administration. It is important to note that CPP alone did not have an effect on netrin-1 receptor expression (i.e. there was no difference in DCC or UNC-5 expression between CPP-saline- and saline-saline-pretreated groups).

DISCUSSION

We have described a series of experiments in which we examined the effects of repeated AMPH exposure on the expression of the netrin-1 guidance cue receptors, DCC and UNC-5, in the adult brain. The main findings that emerge from these studies are that a sensitizing AMPH treatment regimen results in an upregulation of DCC and UNC-5 in the cell body, but not terminal regions, of mesocorticolimbic DA neurons. Moreover, these localized effects of repeated AMPH treatment on netrin-1 receptors appear to depend on glutamatergic neurotransmission, as they are prevented by NMDA glutamate receptor blockade.

It is well-documented that glutamate-mediated neuroadaptations in the VTA underlie the development of sensitization to AMPH. The results presented here raise the intriguing possibility that netrin-1 signaling may participate in the development of sensitization. In support of this idea, we have previously shown that adult *dcc* heterozygous mice, which have reduced levels of DCC but unaltered expression of UNC-5 (Grant et al., 2007), do not develop sensitization to repeated AMPH treatment (Flores et al., 2005). It therefore appears that while repeated AMPH *upregulates* DCC and UNC-5 receptor expression in the VTA, *reduced* DCC expression prevents the development of sensitization. It is important to note, however, that adult *dcc* heterozygous mice also exhibit significant and selective changes in mesocorticolimbic DA function that may contribute to their lack of sensitization. These mice have a small, but significant, reduction in the number of VTA DA neurons compared with their wild-type littermates. They also display exaggerated baseline DA concentrations and

increased expression of TH, but not DA- β -hydroxylase, in the mPFC only. Thus, one possible explanation for why these mice do not develop sensitization to repeated AMPH is that they have an impaired upregulation of netrin-1 receptors in the VTA. Alternatively, the neuroanatomical and neurochemical alterations in the VTA and mPFC may prevent, directly or indirectly, respectively, the development of sensitization to AMPH. These two possibilities are not mutually exclusive. To gain insight into how the AMPH-induced upregulation of VTA netrin-1 receptors may play a role in the development of sensitization, we are currently examining the effects of repeated AMPH treatment on netrin-1 receptor expression in DCC-deficient mice.

While netrin-1 signaling is recognized for its role in organizing neural circuitry in the developing brain, it may be involved in experience-dependent reorganization of neuronal connectivity in the adult brain. Our findings suggest that netrin-1 may be involved in the AMPH-induced reorganization of VTA DA dendritic circuitry recently reported by Mueller et al. (2006). Netrin-1 can attract and repel neurite processes depending on the receptors they express (Barallobre et al., 2005; Furrer et al., 2003; Manitt and Kennedy, 2002; Stein et al., 2001; Suli et al., 2006). Here, we show that both DCC and UNC-5 receptors are highly expressed by adult mesocorticolimbic DA neurons, and that these receptors are upregulated in the VTA by a mild AMPH treatment regimen similar to the one used by Mueller et al. (2006).

Of additional relevance to the present study is the fact that the reported alterations in dendritic structure of VTA DA neurons depend on AMPH-induced increases in VTA expression of the neurotrophic factor, basic fibroblast growth factor (bFGF; Mueller et al., 2006). We have shown, using the exact same protocol as the one used in experiment 3 of this study, that AMPH-induced VTA bFGF expression requires NMDA receptor neurotransmission (Flores et al., 2000). Importantly, we have also demonstrated that AMPH-induced bFGF in the VTA is necessary for the development of sensitization (Flores et al., 2000). Based on these findings we propose that if netrin-1 signaling participates in the development of sensitization to AMPH, it may act in concert with bFGF to promote reorganization of VTA DA circuitry. Both bFGF and netrin-1 can induce reorganization of the actin cytoskeleton via receptor-mediated communication with the Rac1 member of the Rho family of small GTPases, key regulators of the actin cytoskeleton (Li et al., 2002; Shekarabi et al., 2005; Shin et al., 2002, 2004). Furthermore, it has been demonstrated that netrin-1 can induce reorganization of dendritic structure in mature neurons via Rac1 signaling (Nakayama et al., 2000). Thus, it may be that while bFGF signaling promotes DA neurite outgrowth, netrin-1 signaling guides the neurite extension toward its appropriate target. Research in our laboratory is currently exploring if/how bFGF and netrin-1 signaling interact to produce alterations in neuronal morphology.

Changes in netrin-1 receptor expression following repeated exposure to AMPH may also promote AMPA receptor plasticity in VTA neurons. It has been demonstrated that stimulant drugs enhance the ratio of AMPA/NMDA receptor-mediated glutamate neurotransmission in the VTA (Borgland et al., 2004; Boudreau and Wolf, 2005; Faleiro et al., 2004; Saal et al., 2003; Sarti et al., 2007; Ungless et al., 2001), an effect that depends on NMDA receptor neurotransmission at the time of drug treatment (Ungless et al., 2001). It has recently been

shown that activity of the Rac1 Rho GTPase, a downstream netrin-1 effector (Li et al., 2002), enhances excitatory synaptic transmission by promoting AMPA receptor clustering (Wiens et al., 2005). Thus, the upregulation of DCC and UNC-5 receptors in the VTA may be involved in AMPH-induced plasticity of VTA DA circuitry by enhancing the availability of AMPA receptors at the synapse. This effect, in turn, would increase the ability of VTA AMPA receptors to regulate DA transmission in the VTA and NAcc (Giorgetti et al., 2001). Examining a possible connection between netrin-1, Rho GTPases, and AMPA receptor plasticity in the VTA is warranted, as it may be a critical step in transferring sensitization to its maintained expression in the forebrain.

If it is indeed the case that netrin-1 plays a role in the AMPH-induced DA system plasticity that accompanies the development of sensitization, it remains to be determined why we did not observe an effect of AMPH on netrin-1 receptor expression in the NAcc. A possible explanation is that changes in netrin-1 receptor expression are involved in the development, but not the expression of sensitization. For instance, we have demonstrated previously that the same AMPH treatment regimen as the one used in the current study results in an enduring upregulation of bFGF expression in the VTA, but not in DA terminal regions (Flores et al., 1998). Another possibility is that AMPH does induce changes in NAcc netrin-1 receptor expression, but that these changes only become evident at a later time point. Moreover, it may also be the case that guidance cues other than netrin-1 are involved in AMPH-induced alterations in neuronal structure in this terminal region (Yue et al., 1999). In contrast to the lack of effects of AMPH on netrin-1 receptor expression in the NAcc, we observed an increase in mPFC UNC-5 expression in AMPH-pretreated animals administered an AMPH challenge on the sensitization test day, compared with saline-pretreated controls. However, when AMPH-pretreated animals were administered a saline challenge on the test day, this increase was no longer observed. The significance of this finding, which appears to result from a combination of AMPH pretreatment and acute effects of the AMPH challenge on the test day, remains to be determined.

Lastly, it is worth noting that the increase in DCC expression in the saline-AMPH-pretreated group (experiment 3) is of a lesser magnitude compared with the AMPH-pretreated group (experiment 1). In experiment 3, the NMDA glutamate receptor antagonist, CPP, was administered prior to each AMPH pretreatment injection. To control for this additional manipulation, saline-AMPH- and saline-saline-pretreated groups received instead an extra saline injection on each AMPH pretreatment day. The significance of this experimental manipulation lies in the consideration that chronic saline pretreatment in itself can alter the stimulatory effects of AMPH, perhaps by acting as a stressor (Brake et al., 1997). Indeed, both drugs of abuse and stress activate the mesocorticolimbic DA system (Kalivas and Duffy, 1989; Kalivas and Stewart, 1991; Morrow et al., 1993). A variety of stressors, including chronic saline administration, have been found to alter dendritic morphology in cortical and/or subcortical regions (Brown et al., 2005; Dagnino-Subiabre et al., 2005; Liston et al., 2006; Radley et al., 2006; Seib and Wellman, 2003), and it has been recently shown that both drugs of abuse and stress induce similar forms of AMPA receptor plasticity in VTA DA neurons (Fitzgerald et al., 1996; Saal et al., 2003). It may therefore be possible that chronic saline pretreatment, acting as a stressor, regulates the expression of DCC receptors in the VTA. If this is indeed the case, a stress-induced upregulation of DCC

expression in the saline–saline group may be masking the AMPH-induced upregulation of DCC expression in the saline–AMPH group. The hypothesis that stress can regulate the expression of netrin-1 receptors is currently being explored by our group.

CONCLUSION

In summary, we have shown here for the first time that netrin-1 receptor proteins can be regulated in the adult brain, and that they may be involved in AMPH-induced plasticity of mesocorticolimbic DA circuitry. These findings are in agreement with the recent observation that repeated pretreatment with stimulant drugs during adulthood regulates gene expression of a wide variety of guidance molecules (Bahi and Dreyer, 2005; Yue et al., 1999). We have also shown here that the effects of repeated AMPH on netrin-1 receptor expression are mediated by NMDA receptor activation, suggesting that netrin-1 signaling may play a role in the development of AMPH-induced sensitization. Interestingly, NMDA receptor activation is a molecular event known to be involved not only in the development of sensitization, but also in general experience-dependent plasticity in the adult brain. Thus, glutamate-dependent changes in netrin-1 receptor expression in the adult brain may provide the necessary directives for the structural reorganization associated with AMPH-induced sensitization, as well as for other forms of experience-dependent plasticity. These novel findings open exciting avenues for further investigation regarding the role of guidance cues in the adult brain, a subject which we expect will be eagerly explored.

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Abbreviations

AMPH	<i>d</i> -amphetamine sulfate
bFGF	basic fibroblast growth factor
CPP	3-(2-carboxypiperazine-4-yl) propyl-1-phosphonic acid
DA	dopamine
DCC	deleted in colorectal cancer
mPFC	medial prefrontal cortex
NAcc	nucleus accumbens
NMDA	<i>N</i> -methyl-D-aspartate
PBS	phosphate-buffered saline
TH	tyrosine hydroxylase

UNC-5	UNC-5 homologues
VTa	ventral tegmental area

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Fig. 1. Diagram outlining the timing of treatment and experimental procedures in experiments 1 (a), 2 (b), and 3 (c). Sal: saline.

Fig. 2.

Netrin-1 receptor expression by DA neurons in the adult rat. Digitized images of coronal VTA and substantia nigra sections obtained from adult rats under baseline conditions. Both DCC (*top row*) and UNC-5 (*middle row*) were visualized in TH-positive neurons. Most DCC- and UNC-5-positive cells were also TH immunoreactive. Merged high magnification images of VTA sections showing DCC/TH and UNC-5/TH double-labeled neurons (*bottom row*). Scale bars=250 μm (top and middle rows), 50 μm (bottom row).

Fig. 3.

Netrin-1 receptor expression following a sensitizing regimen of AMPH. (a) AMPH-induced locomotor activity during the 90 min test for sensitization. (b) Netrin-1 receptor expression in the VTA of animals pretreated with AMPH or saline. (c) Netrin-1 receptor expression in the NAcc of animals pretreated with AMPH or saline. (d) Netrin-1 receptor expression in the mPFC of animals pretreated with AMPH or saline. Representative examples of Western blot analysis are shown below the graphs. In all bar graphs, the values are expressed as the mean percent of saline pretreated animals (100%). All data are presented as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

Fig. 4. Netrin-1 receptor expression following AMPH pretreatment. (a) Netrin-1 receptor expression in the VTA of animals pretreated with AMPH or saline. (b) Netrin-1 receptor expression in the mPFC of animals pretreated with AMPH or saline. Representative examples of Western blot analysis are shown below the graphs. In all cases, values are expressed as the mean percent of saline pretreated animals (100%). All data are presented as mean $\pm$ S.E.M. * $P < 0.05$, ** $P < 0.01$.

Fig. 5. Alterations in netrin-1 receptor expression following repeated AMPH are mediated by glutamatergic neurotransmission. (a) AMPH-induced locomotor activity during the 90 min test for sensitization in saline-AMPH- and saline-saline-pretreated animals. (b) AMPH-induced locomotor activity during the 90 min test for sensitization in CPP-saline- and CPP-AMPH-pretreated animals. (c) DCC receptor expression in the VTA. (d) UNC-5 receptor expression in the VTA. In all bar graphs, the values are expressed as the mean percent of saline-saline-pretreated animals (100%). All data are presented as mean±S.E.M. ** $P < 0.01$.