Published in final edited form as:

Addict Biol. 2018 May ; 23(3): 857–867. doi:10.1111/adb.12534.

Methamphetamine promotes habitual action and alters the density of striatal glutamate-receptor and -vesicular proteins in dorsal striatum

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Abstract

Goal-directed actions are controlled by the value of the consequences they produce and so increase when what they produce is valuable and decrease when it is not. With continued invariant practice, however, goal-directed actions can become habits, controlled not by their consequences but by antecedent, reward-related states and stimuli. Here we show that pre-exposure to methamphetamine (METH) caused abnormally rapid development of habitual control. Furthermore, these drug-induced habits differed strikingly from conventional habits; we found they were insensitive both to changes in reward value and to the effects of negative feedback. In addition to these behavioral changes, METH exposure produced bidirectional changes to synaptic proteins in the dorsal striatum. In the dorsomedial striatum, a structure critical for goal-directed action, METH exposure was associated with a reduction in glutamate receptor and glutamate vesicular proteins, whereas in the dorsolateral striatum, a region that has previously been implicated in habit learning, there was an increase in these proteins. Together, these results indicate that METH exposure promotes habitual control of action which appears to be the result of bidirectional changes in glutamatergic transmission in the circuits underlying goal-directed and habit-based learning.

Keywords

dorsolateral striatum; dorsomedial striatum; glutamate; goal-directed; habits; methamphetamine

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Author contributions

BWB was responsible for the study concept and design. TMF and RAB acquired and analyzed the data. TMF, LHC and RAB drafted the manuscript. All authors contributed to the interpretation of the data, critical analysis of the manuscript and approved the final version for publication.

Introduction

Drug addiction is a chronic relapsing disorder characterized primarily by a loss of control over drug use. Furthermore, this loss of control can extend well beyond drug use to other behaviors leading to social, legal and financial problems, and importantly, the addicts' behavior often appears to persist despite this negative feedback. Thus, it is becoming increasingly well recognized that, during the development of addiction, exposure to drugs of abuse can alter normal decision-making processes (Everitt & Wolf 2002; Kalivas 2009; Kalivas & Volkow 2011). The observed lack of flexible control over behavior has led to increasing interest in the role of a habit learning process in addiction (Everitt *et al.* 2008; Ostlund & Balleine 2008). Drug exposure has been reported to increase the rate at which habits are acquired, as well as the influence of drug-associated contexts and cues on the performance of drug-related behaviors (Corbit *et al.* 2014a; Corbit *et al.* 2014b; Furlong *et al.* 2015; Nelson & Killcross 2006; Ostlund *et al.* 2010). Under other conditions, habits can be highly adaptive, allowing the control of routine responses to be relegated to a process that requires relatively few cognitive resources and thereby freeing up a limited executive capacity for more demanding tasks (Ostlund & Balleine 2008). However, when habits are driven by drug-use they produce an obstacle for behavioral change and recovery. The loss of executive control over habits may contribute to the way drug-use often persists even in the face of severe negative consequences (American Psychiatric Association 2013; Deroche-Gamonet *et al.* 2004; Hester *et al.* 2013; Vanderschuren & Everitt 2004; Weiss *et al.* 2001).

Although there is growing evidence that drug exposure can accelerate habit formation, the underlying neural changes that lead to these effects are less well understood. Considerable prior evidence has implicated distinct regions of dorsal striatum in aspects of action control; the dorsomedial striatum in the control of goal-directed actions (Corbit & Janak 2010; Pauli *et al.* 2012; Yin *et al.* 2005) and the dorsolateral striatum in the control of habits (Corbit *et al.* 2012; 2014b; Yin *et al.* 2004; Zapata *et al.* 2010). Of note, methamphetamine (METH) has been reported to alter the density of dendritic spines on medium spiny neurons in the striatum, reducing spines in dorsomedial striatum but increasing them in the dorsolateral striatum, an effect that suggests potential changes in glutamate mediated synaptic plasticity in these regions (Jedynak *et al.* 2007). While contextual cues paired with METH can transiently promote habitual responding for food (Furlong *et al.* 2015), it is not known how more extensive METH exposure, shown elsewhere to produce changes in striatal neuronal morphology, impacts instrumental learning more generally and in the absence of specific drug-paired cues. Nevertheless, it is likely that METH, like other psychostimulants, will accelerate habit learning and that this might be due to plastic changes in dorsal striatal regions.

In the current study, we assessed the effect of prior exposure to METH on the subsequent acquisition and control of instrumental actions in rats. Specifically, we compared the sensitivity of drug-induced and overtraining-induced habits to the effects of outcome devaluation and negative feedback. We then evaluated changes in the expression of proteins that comprise the NMDA and AMPA receptors, as well as vesicular glutamate transporters, in the dorsomedial and dorsolateral striatal subregions following METH exposure in order to

begin to understand the neural mechanisms that underlie the persistent behavioral changes produced by METH.

Materials and methods

Subjects and Apparatus

55 male Long-Evans rats from Harlan Industries (Indianapolis, IN) served as subjects in Experiment 1, and 10 male Long-Evans rats (Monash University, Victoria, Australia) served as subjects in Experiment 2. Rats were housed in pairs with ad libitum access to water and food except as described below. All procedures were approved by the animal research committees of either the University of Sydney or UCLA.

The behavioral procedures were performed in chambers enclosed in sound- and light-attenuating shells (Med Associates, East Fairfield, VT). Each chamber was equipped with two retractable levers that could be extended to the left and right of a recessed food magazine. Attached to the food magazine was a pellet dispenser, that delivered a 45 mg sucrose-pellet (Bio-Serv, Frenchtown, NJ). An infrared photobeam crossed the magazine opening, allowing for the detection of head entries. Illumination was provided by a houselight (3 W, 24 V) located on the wall opposite the magazine. Microcomputers running the Med-PC program (Med Associates) controlled all experimental events and recorded lever presses and magazine entries.

Methamphetamine pre-exposure

Rats in Experiment 1 were divided into two groups for the pretreatment phase (METH group: $n=27$; Control group: $n=28$). The procedures followed those of Jedynak et al., 2007, who demonstrated differential effects of METH-exposure on spine density in dorsomedial and dorsolateral striatum. Specifically, the METH group received injections of METH-HCl (Sigma-Aldrich, MO, USA) over 28 days divided into 4 weekly periods of five consecutive daily injections followed by two non-drug days. The doses were escalated across days ranging from 0.5 mg/kg on day 1 to a maximum of 6 mg/kg on day 28. The control group received injections of saline vehicle at the same volume as the METH group (i.e. 1 ml/kg). Lever-press training began 4 weeks after the final treatment and continued for a 4-week period in order to investigate the long-term impact of METH on behavior. Rats in Experiment 2 were treated with either METH or saline following the same protocol, and were sacrificed 8 weeks after the last treatment; i.e., at the time behavioral testing ended for Experiment 1 to examine persistent neuroadaptations following drug exposure (as in Jedynak et al. 2007; Ghasemzadeh et al. 2009a; Loftis & Janowsky 2000). A separate group of animals were used for Experiment 2 to establish the effects of METH exposure on brain protein content in the absence of any effects induced by the behavioral procedures.

Lever press training

Rats were initially food deprived to 90% of their free-feeding weight and fed once per day approximately 2 hrs after the daily training session.

Prior to instrumental training rats were given three magazine training sessions over successive days during which they were placed in the operant chamber for 30 min with the levers retracted and the sucrose-pellets were delivered into the food magazine on a random-time 60 sec schedule. Half of the rats from each drug-treatment group were given extended training (ET) and the remaining half given moderate training (MT). For ET there were 19 days of training. Rats were placed in the operant chamber for 20 min each session, where the lever was inserted and responses on the lever resulted in a single pellet (with left and right levers counterbalanced across groups). On day 1, each press was rewarded (continuous reinforcement; CRF) and then on subsequent days pressing was rewarded using variable interval (VI) schedules that increased across days (VI-15 for days 2–3, VI-30 for days 4–5 and VI-60 for days 6–19, where the pellet was delivered for the first lever press made after an average interval of 15, 30 and 60 seconds, respectively). The remaining animals received MT which was delayed for 12 days so that MT and ET groups completed training on the same day relative to the METH treatment. Following the CRF session, these rats were trained using VI-15, VI-30 and VI-60 schedules (two days each) before testing.

Outcome devaluation and testing

Following training, sensitivity to outcome devaluation was assessed using two separate tests: an extinction test and a reinforced test, after devaluation of the pellet-outcome using a conditioned taste aversion procedure (Yin *et al.* 2004). Each group was divided into devaluation and control conditions and then all rats were placed in individual feeding cages where they were allowed to consume the sucrose-pellets freely for 30 min each day for three days. After removal from the cage each day, the animals received either the devaluation treatment, i.e. an injection of lithium chloride (0.15 M LiCl, 20 ml/kg, i.p.), or the control treatment, i.e. an injection of sodium chloride (0.15 M, 20 ml/kg, i.p.). The amount of pellets consumed in the feeding cages was measured, and the amount consumed on the final day was expressed as a percentage of the amount eaten prior to treatment. The design of this treatment means that only half of the animals experienced lithium chloride allowing comparison of the effects of METH exposure on outcome devaluation to an unpoisoned control. This is consistent with several prior studies that use devaluation by taste aversion as a tool to probe habit learning (e.g. Nelson & Killcross 2006; Yin *et al.* 2004; Tran-Tu-Yen *et al.* 2009).

The next day, lever press performance of all rats was first examined in a 5-min extinction test, where the lever was extended but no pellets were delivered. Finally, a 15-min reinforced test was carried out the next day in which pellets were delivered as a result of lever-pressing on a fixed interval-60 sec schedule. A fixed interval schedule was used to ensure that the number and timing of outcome deliveries in this test was consistent for all animals.

Histology: Western Blotting

Analysis of glutamate receptor and glutamate vesicular protein content was conducted on a second group of rats to examine the impact of METH on plasticity in the medial and lateral divisions of the dorsal striatum. Rats were administered either METH or saline in a manner identical to that of experiment 1, and protein content examined 8 weeks later to coincide with the test period in experiment 1.

Rats were overdosed with sodium pentobarbital and the brains rapidly removed and placed briefly in ice-cold saline solution. Two coronal blocks of tissue were then cut using an ice-cold brain matrix, and the lateral and medial dorsal striatum were dissected (approx. + 1.70 mm and +0.70 mm anterior to bregma for DLS and +0.70 mm and -0.30 mm anterior to bregma for DMS; Paxinos & Watson 2005). This coordinate corresponds with posterior DMS which has consistently been implicated in both the acquisition and expression of goal-directed behavior using both lesion and inactivation techniques. Previous results regarding the anterior DMS are mixed, but compensation may occur during the extended training of response-outcome associations when lesions are used, and spread of drug cannot be ruled out when inactivation techniques are used (compare Corbit & Janak 2010 and Yin *et al.* 2005). Given the ambiguity of these results we focused on the posterior DMS. The tissue was immediately frozen on dry ice and then stored at -80°C until use.

Protein content was examined in the synaptosomal membrane fraction, which was obtained using a fractionation procedure modified from Ghasemzadeh, *et al.* 2009a and used previously in Furlong *et al.* 2016. The synaptosomal fraction, rather than the whole tissue fraction, was examined as it is altered by cocaine exposure across a number of brain structures, and represents receptors on the surface of the cell membrane available to influence cellular activity (Ghasemzadeh *et al.* 2009a; Ghasemzadeh *et al.* 2009b). Briefly, the tissue was lysed for 1 hr in 4mM HEPES solution containing 0.32M sucrose and 1% protease inhibitor, and then centrifuged (1000g for 10 min, and the resulting supernatant centrifuged at 10 000g for 15 min). Next, the pellet was resuspended in 4mM HEPES solution, and then centrifuged (10 000g for 15min) and lysed hypo-osmotically (30 min) before again centrifuging (21 000g for 30 min) and resuspending in 4mM HEPES solution.

Samples were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gels under reducing conditions (10ug/sample, determined using a BCA protein assay, Thermo Scientific-Pierce, Rockford, IL, USA). Proteins were then transferred onto polyvinylidene fluoride membranes using a semi-dry transfer apparatus (Biorad, Hercules, CA, USA) and then blocked in 5% non-fat milk power in 0.1M tris-buffered saline containing 0.1% Tween-20 (M-TBS-T, 1 hr, RT). The membranes were then incubated in a primary antibody (M-TBS-T, 24 hr, 4°C), and then in a HRP-secondary antibody (for 2 hr, RT) which were all obtained from EMD-Millipore (MA, USA) unless otherwise stated. The primary antibodies were for the NMDA receptor subunits: rabbit-anti-NR1 (cat#AB9864, 1:1000; 120kDa), rabbit anti-NR2A (cat#05-901R, 1:10 000; 170kDa), and mouse-anti-NR2B (cat#05-920, 1:1000; 180kDa); the AMPA-selective glutamate receptors: mouse-anti-GluR1 (cat#MAB2263, 1:1000; 110kDa) and rabbit-anti-GluR2 (cat#Ab1768, 1:1000; 110kDa); the vesicular glutamate transporters: mouse-anti-VGLUT1 (cat#MAB5502, 1: 20 000; 62kDa) and guinea-pig anti-VGLUT2 (cat# AB2251, 1: 20 000; 52kDa); and finally the protein-loading control: rabbit-anti- $\alpha\beta$ -tubulin (Cell Signalling, cat#sc-2020, 1:1000; 52kDa). The corresponding secondary antibodies were either goat-anti-guinea-pig, goat-anti-mouse, or goat-anti-rabbit (all at 1:20 000).

Finally, a chemiluminescence method was used to visualise each blot on Kodak photographic paper (Amersham ECL Prime Detection Reagent, GE Healthcare). The

photographs were then scanned and the band density of each protein quantified using Image-J software and corrected against the $\alpha\beta$ -tubulin loading control.

Statistical analysis

One-way and repeated measures ANOVA was used to analyze body weight and behavioral training data. Extinction and reinforced tests were analyzed using two-way ANOVAs where drug-treatment (METH versus saline treatment) and devaluation treatment (devalued versus control conditions) served as the two factors. Simple effects analysis were then used to examine any significant interactions. Western blot data was analyzed using separate one-way ANOVAs for each protein examined.

Results

Body Weight, Instrumental Training and Devaluation

At the commencement of the studies and prior to any treatment, body weight did not differ between the saline and METH treated groups (328 ± 4 and 335 ± 9 g, respectively, $F_{1,54} < 1$, $p > 0.1$). However, at the end of the 4-week treatment period rats in the METH group weighed significantly less than those in the saline group (388 ± 6 versus 365 ± 6 g, $F_{1,54} = 6.1$, $p < 0.05$). Importantly, 4 weeks after the final treatment session, corresponding to the time that lever-training commenced, there were no longer any differences between groups, suggesting that the body weight of the METH group recovered during this period (454 ± 8 and 447 ± 6 g, saline and METH groups respectively, $F_{1,54} < 1$, $p > 0.1$).

All rats acquired the instrumental response during training. Lever pressing rate increased for all groups from day 1 to day 7 as the training schedule increased from CRF through to VI60 (Fig. 1A). A repeated measures ANOVA confirmed that response rates increased across training for both MT and ET groups ($F_{1,156} = 58.5$, $p < 0.01$ and $F_{1,162} = 34.9$, $p < 0.01$, respectively), but there were no significant differences in response rates between groups ($F_{1,26} = 1.6$, $p > 0.1$ and $F_{1,27} = 1.2$, $p > 0.1$, respectively), and no interactions ($F_{1,156} < 1$, $p > 0.1$ and $F_{1,162} = 1.9$, $p > 0.1$, respectively). An ANOVA revealed no differences among the four groups on day 7 of training which was the final day of training for the MT groups ($F_{3,51} = 1.97$, $p > 0.1$). There was also no difference between the two ET groups on their final day of training on day 19 ($F_{1,27} < 1$, $p > 0.1$).

Following lithium chloride treatment, pellet consumption was decreased for the devalued condition compared to the control condition regardless of Saline or METH treatment (Fig. 1B). There was a main effect of devaluation ($F_{1,51} = 230$, $p < 0.01$), with no effect of drug treatment ($F_{1,51} < 1$, $p > 0.1$) and no interaction ($F_{1,51} < 1$, $p > 0.1$).

Outcome devaluation under extinction conditions

As shown in Fig. 1C, animals that underwent MT and were treated with saline were sensitive to the devaluation treatment, i.e. animals that received devaluation treatment displayed reduced rates of responding compared to animals that received the control treatment. However, MT groups that were treated with METH did not show this sensitivity as equal rates of responding were shown for the devaluation and control treatment groups. Statistical

analysis confirmed these observations. There was no main effect of devaluation condition ($F_{1,26} = 2.6$, $p > 0.1$) or drug treatment ($F_{1,26} < 1$, $p > 0.1$), but there was a significant devaluation condition by drug treatment interaction ($F_{1,26} = 4.3$, $p < 0.05$). Post-hoc analysis confirmed that there was a difference between rates of responding for the devalued and control conditions for the Saline group ($F_{1,26} = 7.4$, $p < 0.05$), but not for the METH group ($F_{1,26} < 1$, $p > 0.1$).

Overtraining is known to produce insensitivity to devaluation (Adams 1982; Ostlund & Balleine 2008; Yin *et al.* 2004). Consistent with these studies, neither of the ET METH or Saline groups showed differences in their rates of responding under devalued and control conditions (Fig. 1D). There was no main effect of group ($F_{1,27} < 1$, $p > 0.1$), no main effect of devaluation ($F_{1,27} < 1$, $p > 0.1$) and no interaction between these factors ($F_{1,27} < 1$, $p > 0.1$).

Outcome devaluation with feedback

Because the lack of sensitivity to the negative consequences of one's behavior is a major feature of addiction syndromes (American Psychiatric Association 2013), we next examined whether responding would persist in METH-treated animals despite negative feedback from the now-devalued outcome. To test this, we adopted a punished responding paradigm whereby animals now received the devalued outcome following lever pressing. This procedure has been shown elsewhere to return habitual responding to goal-directed control (Adams 1982; LeBlanc *et al.* 2013; Ostlund & Balleine 2008). When lever-pressing was reinforced during the test, saline treated animals in both the MT (Fig. 1E) and ET (Fig. 1F) groups demonstrated sensitivity to the devaluation treatment, reducing responding under devalued versus control conditions. In contrast, the METH treated animals in both ET and MT training groups remained insensitive to devaluation, responding at equivalent rates for both conditions. For MT analysis, there was a main effect of devaluation condition ($F_{1,26} = 7.0$, $p < 0.05$) and a main effect of drug treatment ($F_{1,26} = 5.1$, $p < 0.05$) and for ET analysis there was no main effect of devaluation ($F_{1,27} < 1$, $p > 0.1$) or drug treatment ($F_{1,27} = 4.0$, $p > 0.05$). More importantly, there was a significant interaction between drug treatment and devaluation condition for both MT ($F_{1,26} = 6.6$, $p < 0.05$) and ET ($F_{1,27} = 5.8$, $p < 0.05$) analyses. Post-hoc analysis confirmed there were differences between rates of responding for the devalued and control conditions for the saline-treated group ($F_{1,26} = 15.5$ and $F_{1,27} = 4.3$, $p < 0.05$ in both cases), but not for the METH-treated group ($F_{1,26} < 1$ and $F_{1,27} = 2.6$, $p > 0.1$ in both cases).

Histological analyses

Prior studies have demonstrated that distinct regions of the dorsal striatum are essential for the acquisition and expression of goal-directed versus habitual instrumental responding. Specifically, lesions and/or inactivation of the dorsomedial striatum interferes with the acquisition and expression of goal-directed actions (Corbit & Janak 2010; Pauli *et al.* 2012; Yin *et al.* 2005), and lesions or inactivation of the dorsolateral striatum interferes with the development and expression of habitual actions (Furlong *et al.* 2014; Yin *et al.* 2004; Zapata *et al.* 2010). Further, recently Jedynak *et al.* (2007) reported an increase in spines in dorsolateral striatum, and decrease in spines in dorsomedial striatum following a schedule of

METH treatment similar to that used in the current study. For these reasons, we examined glutamate associated proteins located in these regions using Western blot.

Western blot analysis

As neural activity in the striatum is driven predominately by excitatory glutamatergic input, we examined changes in expression of proteins associated with glutamate-transmission in dorsal striatum. Example blots for each protein are shown in Fig. 2A. Specifically, we examined the density of N-methyl-D-aspartate (NMDA) receptor proteins, which consist of an obligatory NR1 subunit, and at least one other subunit (e.g. NR2A or NR2B) and the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor proteins, which consist of a GluR2 subunit, and one other subunit (most commonly GluR1). We also examined the most prevalent vesicular glutamate transport proteins (VGLUT1 and VGLUT2), which regulate the amount of glutamate packaged into synaptic vesicles in the presynaptic terminal.

Dorsolateral Striatum—The results of the protein analysis for DLS are shown in Fig. 2B. It can be seen from inspection of the figure that the density of the examined proteins tended to be greater following METH exposure than following saline exposure. Statistical analysis confirmed that these differences were significant for the NMDA receptor subunits, with both the NR1 and NR2A receptor subunits significantly greater for the METH group compared to the Control group (NR1: $F_{1,8} = 10.171$, $p < 0.05$; NR2A: $F_{1,8} = 6.439$, $p < 0.05$). The NR2B receptor subunit was not examined for DLS, due to lack of sufficient sample.

The AMPA receptor subunits also tended to be greater for the METH group compared to the Control group in the DLS. However, only the difference between the GluR1 receptor subunits, and not the GluR2 receptor subunits, reached statistical significance (GluR1: $F_{1,8} = 12.135$, $p < 0.01$; GluR2: $F_{1,8} = 3.269$, $p = 0.108$). Finally, the vesicular glutamate transporters also tended to be greater for the METH group compared to the Control group in the DLS. Statistical analysis revealed that these differences were significant for only VGLUT2, and not for VGLUT1 (VGLUT2: $F_{1,8} = 5.808$, $p < 0.05$; VGLUT1: $F_{1,8} = 3.75$, $p = 0.089$).

Dorsomedial Striatum—During the dissection of the DMS, insufficient sample was obtained from one animal in the Control group resulting in the analysis of 4 samples for the Control group, and 5 samples for the METH group. The results of the analysis are shown in Fig. 2C. It can be seen from the figure that the density of glutamate-related proteins in the DMS tended to be reduced following METH exposure compared to saline exposure, in contrast to the DLS where these proteins tended to be increased. Specifically, statistical analysis confirmed significantly less NR2A and NR2B receptor subunits for the METH group compared to the control group in the DMS (NR2A: $F_{1,7} = 5.857$, $p < 0.05$; NR2B: $F_{1,7} = 5.677$, $p < 0.05$). However, there was no such difference between groups for the NR1 receptor subunit ($F_{1,7} = 1.931$, $p > 0.1$). There was also no statistically significant difference between groups in the DMS for either AMPA receptor subunit (GluR1: $F_{1,7} = 1.233$, $p > 0.1$; GluR2: $F_{1,7} = 1.743$, $p > 0.1$). Finally, both VGLUT1 and VGLUT2 were significantly

reduced for the METH group compared to the Control group (VGLUT1: $F_{1,7} = 6.212$, $p < 0.05$; VGLUT2: $F_{1,7} = 11.163$, $p < 0.05$).

Discussion

A growing body of work shows that goal-directed performance can be altered by a history of exposure to potent rewards including drugs of abuse (Corbit *et al.* 2014a; Furlong *et al.* 2014; Nelson & Killcross 2006). Here we show that while METH-treated rats readily acquire a food-motivated instrumental task, their performance was subsequently insensitive to outcome devaluation at a time when saline-treated control animals displayed sensitivity to devaluation and thus evidence of goal-directed performance under the same conditions (i.e. moderate training). This acceleration in the acquisition of a habit suggests that METH exposed animals do not rely on the previously trained response-outcome relationship, or are unable to update the representation of the current value of the earned outcome in order to direct responding, consistent with previous studies utilizing other stimulant drugs such as cocaine and d-amphetamine (Corbit *et al.* 2014a; LeBlanc *et al.* 2013; Nelson & Killcross 2006; Nordquist *et al.* 2007). Furthermore, this insensitivity to devaluation was not only observed under extinction conditions, but also when lever-press was reinforced by contingent delivery of the devalued pellet-outcome. In contrast, whereas extended training produced habitual responding in saline-treated animals under extinction conditions, under reinforced conditions these animals were able to use feedback provided by direct contact with the now-devalued outcome and demonstrated goal-directed responding. Our findings are consistent with prior studies that demonstrate restoration of goal-directed behavior after extended training when negative feedback is applied after an action by the delivery of a devalued outcome (Adams 1982; Ostlund & Balleine 2008) and a failure of this feedback when animals have been previously exposed to a cocaine or METH-paired context (Furlong *et al.* 2015; LeBlanc *et al.* 2013). The resistance to feedback provided by the delivery of a 'punishing' unpalatable food in drug-exposed animals suggests that the resulting habit differs from that produced by simple over-training (LeBlanc *et al.* 2013). 'Normal' habits are proposed to be modifiable by response-contingent punishment, unlike habits that develop from drug-exposure which are persistent and compulsive (Deroche-Gamonet *et al.* 2004; Ostlund & Balleine 2008; Vanderschuren & Everitt 2004).

Importantly, these results are not readily explained by a failure of the devaluation procedure per se as consumption of the pellets when freely available was dramatically reduced for the devaluation groups regardless of prior drug history. Further, we observed no group differences in the rates of responding during training, similar to when amphetamine is administered prior to training also using interval schedules (Nordquist *et al.* 2007). Finally, despite the lower body weight of the METH-treated group compared to the Saline group at the end of the drug-treatment period, there were no such differences in body weight at the time at which training commenced. Thus, habitual performance by the METH group cannot easily be accounted for by insensitivity to conditioned taste aversion, or other motivational factors that might be affected by METH.

To further understand the neural mechanisms that underlie the behavioral changes produced by METH, we examined changes in the level of expression of proteins involved in glutamate

signaling in the dorsal striatum. Specifically, we examined the density of glutamate receptor subunits and vesicular transport proteins in synaptosomal tissue fractions, which contain proteins located at both the pre- and post-synaptic membranes. Given that the majority of neurons in the dorsal striatum are medium spiny neurons (MSN; up to 97.7%; Tepper *et al.*, 2004), our analysis would predominately reflect changes in proteins at MSN synapses. We demonstrated that METH exposure increased the expression of NMDA (NR1 and NR2A) and AMPA (GluR1) receptor subunits in the DLS. In addition, VGLUT2 was also increased in DLS. In contrast, there was a corresponding reduction in NMDA receptor subunits (NR2A and NR2B) in the DMS, as well as a reduction in VGLUT1 and VGLUT2. Hence, METH exposure differentially impacted MSN synapses across the sub-regions of the dorsal striatum.

NMDA and AMPA receptors are associated with fast excitatory transmission, and have been implicated in synaptic plasticity involving glutamatergic afferents to dorsal striatum (Lovinger 2010). The VGLUTs regulate the amount of glutamate packaged into synaptic vesicles and synaptic glutamate release (Fei *et al.* 2008; Moriyama & Yamamoto 2004). Hence, our findings suggest that METH exposure increases glutamate signaling in the DLS, and reduces glutamate signaling in the DMS. The excitatory input that drives neural activity in the dorsal striatum arises predominately from cortex and thalamus, but also from amygdala and hippocampus, via direct synaptic contact with spines on the distal dendrites of MSNs (Smith & Bolam 1990; Voorn *et al.* 2004). The inputs from cortex and thalamus utilize VGLUT1 and VGLUT2 at these synapses, respectively (Kaneko & Fujiyama 2002; Ni *et al.* 1995). Therefore, our findings are suggestive of increased excitatory influence of thalamostriatal pathways over DLS function, and decreased influence of corticostriatal and thalamostriatal pathways over DMS function, and as a consequence, an increased capacity of the DLS to exert control over behavior. Given the role of these regions in promoting habitual and goal-directed action control, respectively, we hypothesize that METH exposure results in the changes in the level of expression of glutamate-related proteins in the dorsal striatal neurons that facilitate the development of habitual behavior.

Importantly, the changes in the level of proteins persisted well-beyond the period of METH exposure (i.e. 8 weeks after the last METH exposure), and therefore provides a potential neural mechanism for mediating long-lasting alterations in behavior. Prior studies have shown that VGLUT1 and NR1 subunits, as well as NMDA-receptor mediated currents, are increased 24 hrs following METH exposure in DLS (Kerdsan *et al.* 2009; Mark *et al.* 2007; Moriguchi *et al.* 2002; Nishino *et al.* 1996; Shirai *et al.* 1996), but the impact of METH following longer abstinence periods has not been examined using sensitizing doses of METH. However, persistent alterations to striatal dendritic spines, the site of excitatory synaptic inputs, have been reported in dorsal striatum 3 months after METH exposure, suggesting potential changes to glutamate-mediated plasticity (Jedynak *et al.* 2007). Consistent with the current study, Jedynak and colleagues showed that METH exposure reduced spine density in DMS, but increased spine density in DLS, consistent with the decreased and increased glutamate-related proteins that we observed in these regions, respectively (Jedynak *et al.* 2007). Importantly, both mushroom and thin spines were increased in DLS (Jedynak *et al.* 2007). As these types of spines are known to contain functional AMPA and NMDA receptors, respectively (Matsuzaki *et al.* 2001), it would be

expected that there would be a corresponding increase in these receptors in the DLS, as was demonstrated in the current study. Together these studies suggest that METH exposure results in rapid up-regulation of glutamate transmission in DLS that is maintained with repeated drug-administration and across extended abstinence periods. In turn, long-lasting plasticity in glutamate transmission is likely to contribute to habitual actions resulting from METH exposure.

The importance of striatal glutamate transmission in the development of habitual instrumental actions is demonstrated when the expression of habitual responding is blocked by administration of glutamate receptor antagonists. Specifically, AMPA receptor antagonists have been used to prevent habitual action following exposure to ethanol and high-calorie food, and an NMDA receptor antagonist has been used to remove a habit bias, when administered into DLS (Corbit *et al.* 2014b; Furlong *et al.* 2014; Pauli *et al.* 2012). Together, these studies suggest that a similar manipulation of DLS might also prevent habitual action following METH exposure. This line of investigation would be important for demonstrating a causal link between the increase in AMPA and NMDA receptor subunits in DLS and habitual action associated with METH exposure in the current study. Several other studies also show increased activity of the glutamate system in DLS for other drugs of abuse that accelerate the transition to habits, such as cocaine, amphetamine and alcohol which further suggest that there may be a causal relationship between glutamate alterations in the DLS and habits. For example, an increase in spines in DLS has been reported following extensive alcohol consumption or amphetamine exposure, which was accompanied by increased glutamate transmission in DLS (Cuzon Carlson *et al.* 2011; Li *et al.* 2003). Of note, the increased spines following amphetamine exposure were confined to the distal, rather than proximal dendrites, which are preferentially innervated by extrinsic glutamatergic inputs (Smith & Bolam 1990) again indicating increased control of DLS by excitatory inputs. Further, NR1, NR2B, NR2A and GluR1 subunits are persistently increased in DLS (for 2–4 weeks) following chronic cocaine exposure (Ghasemzadeh *et al.* 2009a; Ghasemzadeh *et al.* 2009b; Loftis & Janowsky 2000). Thus, drugs of abuse, that accelerate habitual actions, tend to increase glutamatergic-related transmission within the DLS suggesting a common neural mechanism may underlie the observed behavioral changes.

On the other hand, it is likely that changes to DMS resulting from METH exposure are also important for the development of habitual behavior. We have recently shown that DMS-specific manipulations using the adenosine 2A receptor antagonist, ZM241385, were effective in rescuing animals' sensitivity to negative feedback in a METH-paired context (Furlong *et al.* 2015). Hence, it is possible that manipulation of activity of the glutamate system within DMS may also be sufficient, or even necessary, to restore goal-directed behavior following METH exposure in the current study. To this end, we have shown previously that changes in glutamate activity in DMS following cocaine exposure are important for behavioral control. Specifically, we rescued goal-directed behavior in animals exposed to cocaine using N-acetylcysteine, a regulator of glutamate-cysteine exchange, which also restored glutamate homeostasis in DMS (Corbit *et al.* 2014). Therefore, it is likely that METH exposure not only directly influences goal-directed control through changes in DMS activity, but such changes likely reflect alterations in glutamatergic activity and not just glutamate receptor protein levels.

The role of glutamate plasticity in the development of drug-addiction has become increasingly well-recognized over the past decade (Everitt & Wolf 2002; Gass & Olive 2008; Kalivas 2009; Kalivas & Volkow 2011; Tzschentke & Schmidt 2003). A particular focus has been the cortical input to the nucleus accumbens core and shell, which make up the ventral striatum, with far less focus on dorsal striatum. Changes to glutamate signaling in the ventral striatum have been proposed to underlie the alterations in behavior that promote addiction, including relapse to drug-seeking, drug-craving, compulsive drug-seeking and habitual behavior (Everitt & Wolf 2002; Gass & Olive 2008; Kalivas 2009; Kalivas & Volkow 2011; Tzschentke & Schmidt 2003). Our current research adds to the recent accumulation of evidence that exposure to drugs of abuse affects glutamate signaling in the dorsal striatum in a region-specific manner. Hence, in addition to the ventral striatum, persistent alterations in glutamatergic inputs to the dorsal striatum are also likely to underlie addiction-related behaviors, especially those related to the facilitation of habitual behavior.

In summary, we have extended previous studies demonstrating that prior stimulant treatment leads to the rapid development of habitual actions that are resistant to negative feedback, and our histological analyses tie this alteration in the determinants of behavior to bidirectional changes in glutamatergic transmission in the dorsomedial and dorsolateral striatum following METH treatment.

Acknowledgments

The research reported in this paper was supported by grants from the NICHD, #HD59257; the NIAAA, #AA018014, and from the NHMRC of Australia; GNT 633268, to BWB. BWB was supported by a Senior Principal Research Fellowship from the NHMRC; GNT1079561. We would like to thank Aedan Roberts for his assistance with Western blotting.

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Fig. 1.

(A). Training: Rates of lever-pressing during training (7 days for the moderate training groups, MT, and 19 days for the extended training groups, ET). There were no differences between the saline or METH treated groups on day 7 of training, nor on day 19 of training. (B). Consumption: Percentage of pellets consumed on the 3rd day of free-feeding compared to the 1st day following lithium chloride pairings (deval) or following saline pairings (control). Both Saline and METH treated animals reduced consumption of the pellets following devaluation. (C). MT-Extinction: Rates of responding during extinction test for moderate training groups. The saline treated group demonstrated goal-directed responding reducing responding on the lever when it was associated with a devalued outcome, compared to when it was associated with a non-devalued outcome (control condition). In contrast, the METH treated animals pressed the lever at equal rates under both conditions, and thus demonstrated habitual responding. (D). ET-Extinction: Rates of responding during extinction test for extended training groups. Both the saline and METH treated groups demonstrated habitual responding pressing the lever at equal rates under control and devalued conditions.

following extended training. (E). MT-reinforced: Rates of responding during reinforced test for moderate training groups. When the pellet was delivered contingent on lever pressing, the METH treated group continued to respond in a habitual manner despite feedback on their actions. (F). ET-reinforced: Rates of responding during reinforced test for extended training groups. When the pellet was delivered contingent on lever pressing, the saline treated group reverted to goal-directed responding, but the METH group continued to respond at equal rates under both control and devalued conditions.

Fig. 2.

(A). Examples of Western blots. Representative blots of each protein examined in the DLS for the saline and METH treated groups. NR1 (120 kDa), NR2A (170 kDa), GluR1 (110 kDa), GluR2 (110 kDa), VGlut1 (62 kDa) and VGlut2 (52 kDa). Protein density was corrected by beta-actin loading control. (B). Density of proteins in DLS. Compared to the saline treated group, the METH treated group tended to have increased density of glutamate-associated proteins. Differences were statistically significant for NR1, NR2A, GluR1 and VGlut2, but not GluR2 or VGlut1. (C). Density of proteins in DMS. Compared to the saline treated group, the METH treated group tended to have reduced density of glutamate-associated proteins. Differences were statistically significant for NR2A, NR2B, VGlut1 and VGlut2, but not NR1, GluR1 or GluR2.