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Comparison of Systemic and Local Methamphetamine Treatment on Acetylcholine and Dopamine Levels in the Ventral Tegmental Area in the Mouse

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Abstract

Acetylcholine (ACh) is an important mediator of dopamine (DA) release and the behavioral reinforcing characteristics of drugs of abuse in the mesocorticolimbic pathway. Within the ventral tegmental area (VTA), the interaction of DA with ACh appears to be integral in mediating motivated behaviors. However, the effects of methamphetamine on VTA ACh and DA release remain poorly characterized. The current investigation performed microdialysis to evaluate the effects of methamphetamine on extracellular levels of ACh and DA. Male C57BL/6J mice received an IP injection (saline, 2 mg/kg, or 5 mg/kg) and an intra-VTA infusion (vehicle, 100 μ M or 1 mM) of methamphetamine. Locally perfused methamphetamine resulted in no change in extracellular ACh compared to vehicle, but caused a strong, immediate and dose-dependent increase in extrasynaptic DA levels (1240% and 2473% of baseline, respectively) during the 20-minute pulse perfusion. An IP injection of methamphetamine increased extrasynaptic DA to 275% and 941% of baseline (2 mg/kg and 5 mg/kg, respectively). Systemic methamphetamine significantly increased ACh levels up to 275% of baseline for 40 – 60 minutes (2 mg/kg) and 397% of baseline for 40 – 160 minutes (5 mg/kg) after injection. ACh remained elevated above baseline for 2 to 3 hours post injection, depending on the methamphetamine dose. Methamphetamine-induced locomotor activity was dose-dependently correlated with extrasynaptic VTA ACh, but not DA levels. These data suggest that methamphetamine acts in the VTA to induce a robust and short-lived increase in extracellular DA release but acts in an area upstream from the VTA to produce a prolonged increase in ACh release in the VTA. We conclude that methamphetamine may activate a recurrent loop in the mesocorticolimbic DA system to stimulate pontine cholinergic nuclei and produce a prolonged ACh release in the VTA.

Keywords

Psychostimulants; Microdialysis; Mesolimbic System; Pontine Tegmentum; Addiction; Reward

The midbrain dopamine cell groups A9 and A10 provide major ascending dopaminergic tone to the telencephalon and have been implicated in a wide variety of tasks (Wise, 1982, Oades and Halliday, 1987, German and Manaye, 1993, Robbins and Everitt, 1996, Schultz, 2006).

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The A9 cell group, thought to be involved in initiating motor behaviors, projects from the substantia nigra (SN) to the dorsolateral striatum in rodents (German and Manaye, 1993) and collateral projections in primates reach dorsolateral prefrontal cortex (Porrino and Goldman-Rakic, 1982). Similarly, the A10 cell group within the ventromedial mesencephalon, often referred to as the ventral tegmental area (VTA), has dense mesocortical and mesolimbic connections that terminate in the nucleus accumbens (NAc), medial prefrontal and temporal cortices and basal forebrain structures such as the amygdala, bed nucleus of the stria terminalis and lateral septum (Hasue and Shammah-Lagnado, 2002). These connections collectively form the mesocorticolimbic pathway and play a critical role in diverse motivational processes including procuring and consuming food, engaging in sexual behavior, and addiction-like behaviors (Wise, 1982, Koob, 1992, Robinson, 2004, Salamone et al., 2007). Since drug addiction is often characterized as a dysfunction of motivational processes it is not surprising that the majority of addiction research has focused on the role of DA within the structures of the mesocorticolimbic system (Koob and Le Moal, 1997, Di Chiara et al., 1999, Wise, 2004, Baler and Volkow, 2006).

Within the VTA, DA neurons fire in response to novel stimuli, conditioned stimuli and non-predicted rewards (Fiorillo et al., 2003, Schultz, 2007). Different firing frequencies determine the rate of postsynaptic DA release and enable various behaviors. Tonic pacemaker firing maintains synaptic DA within a narrow concentration range and is associated with movement, cognition and motivation. Phasic burst firing results in rapid increases in synaptic DA and enables reward prediction error. Drugs that affect the burst firing of DA neurons and synaptic concentrations of DA can ultimately modify stimulus processing and reward-related behaviors (Kalivas, 1993, Schultz, 2006).

Several neurotransmitters, such as noradrenaline, glutamate, GABA, and ACh modulate DA cell firing and DA release from dendrites and in terminal fields (Lacey et al., 1987, Mereu et al., 1987, Johnson and North, 1992, Kalivas, 1993, Moghaddam and Bolinao, 1994, Bardo, 1998, Zhang et al., 2002, Paladini et al., 2004, Schultz, 2006). The VTA receives cholinergic innervation from the pedunculopontine (PPTg) and laterodorsal tegmental (LDTg) nuclei which modulate DA cell firing and release via nicotinic and muscarinic ACh receptors on DA neurons and dendrites (Calabresi et al., 1989, Yeomans et al., 2001, Floresco et al., 2003). The intra-VTA interaction of DA with ACh appears to be integral in mediating motivated behaviors. Cholinergic agonists passively or actively infused into the VTA stimulate increases in DA release in the NAc and prefrontal cortex (PFC) (Westerink et al., 1996, Westerink et al., 1998, Gronier et al., 2000) and are reinforcing, as they support self-administration and conditioned place preference (Ikemoto and Wise, 2002, Ikemoto and Wise, 2004), brain stimulation reward (Redgrave and Horrell, 1976, Yeomans and Baptista, 1997, Yeomans et al., 2001) and the acquisition of food reinforced self administration (Sharf et al., 2006). The rewarding and locomotor activating properties of commonly abused psychostimulants such as cocaine (Carrigan and Dykstra, 2007b), amphetamine (Gerber et al., 2001) and methamphetamine (Taguchi et al., 1998) are in part mediated by DA-ACh interactions within the VTA and NAc.

The locomotor stimulant properties of methamphetamine are a valuable correlate to its effects on the neurochemical environment. Some of the neurochemical substrates and anatomy underlying psychostimulant-induced locomotor activity are shared with those that mediate their reinforcing effects as well. The characteristic increase in dopaminergic transmission and firing between the VTA and NAc that facilitates the reinforcing effects of drugs also contributes to appetitive behaviors and sensitization of the locomotor stimulant response (Wise and Bozarth, 1987). Several studies highlight the importance of DA underlying the behavioral stimulation of amphetamine (Kelly and Iversen, 1976, Clarke et al., 1988). The DA receptor subtypes D1 and D2, specifically, have been implicated in mediating acute amphetamine-

(Narayanan et al., 1996, Xu et al., 2000) and cocaine-induced (Karasinska et al., 2005) locomotor activity.

Methamphetamine is a powerful behavioral reinforcer that supports self-administration and drug- and cue-induced reinstatement behavior in mice (Yan et al., 2006). Nevertheless, the effects of methamphetamine on DA release and VTA cholinergic tone remain unknown. The current investigation evaluated extracellular levels of ACh and DA in the VTA in response to methamphetamine. We first examined the locus of methamphetamine effects by administering methamphetamine directly into the VTA using reverse microdialysis. Next we investigated the effects of systemic methamphetamine injection on somatodendritic DA and ACh release in the VTA. The final experiment assessed prolonged locomotor activity following administration of systemic methamphetamine.

Experimental Procedures

Animals

Male C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, ME) were used in this study. Animals were experimentally naïve and between 8 and 13 weeks old for all experiments. Mice were group housed until being surgically implanted with guide cannulae, after which they were singly housed. For the first two experiments, animals were housed in a temperature and humidity controlled vivarium on a 12h:12h light-dark cycle with lights on at 0700h. For the third experiment, housing and procedures were carried out in the Portland VA Medical Center in a temperature and humidity controlled vivarium on a 12h:12h light-dark schedule. Food and water were available *ad libitum* throughout the study, except during the experimental procedures. Animal husbandry conformed to the guidelines set forth in the National Research Council of the National Academies publication regarding the principles of laboratory animal care (National Research Council of the National Academies, 2003) and in a manner approved by the University's Institutional Animal Care and Use Committee at Oregon Health & Science University and the Portland VA Medical Center's Institutional Animal Care and Use Committee.

Drugs

Methamphetamine hydrochloride was obtained from the National Institute on Drug Abuse drug supply program (Research Triangle Institute, Research Triangle Park, NC). Neostigmine hydrobromide was purchased from Sigma (St. Louis, MO).

Surgery

Mice were anesthetized with isoflurane (2% in O₂) and unilaterally implanted with a stainless steel guide shaft (21-gauge, 5 mm long) positioned 3.25 mm above the VTA. Coordinates for guide shaft placement were AP, -2.92 mm from bregma; L, 0.6 mm from midsagittal suture; and V, 1.3 mm from leveled skull surface (Paxinos and Franklin, 2001). Guide shafts were implanted using a Cartesian stereotaxic device (Kopf Instruments) and were secured with dental acrylic along with two, 000-120 cap screws (one anterior to bregma and one posterior to lambda) that served to anchor the dental acrylic to the skull. Patency of guide shafts was maintained throughout the study by inserting a 26-gauge stainless steel wire stylet. Mice were allowed at least one week to recover prior to testing.

In Vivo Microdialysis

One day before testing, a 7.5 mm long microdialysis probe was inserted into the guide shaft so that it extended 2.5 mm beyond the guide shaft into the VTA. Microdialysis probes were constructed in a concentric design described previously (Hoebel et al., 1991). This design

consisted of a 10.5 mm long, 26-gauge stainless-steel tube surrounding a fused silica glass tube (75 μm i.d. $\times$ 152 μm o.d., 30 cm long; Polymicro Tech Inc., Phoenix, AZ) with a 0.75 mm long tip of dialysis membrane (218 μm o.d., 9000 MW cutoff; Spectrum Med. Co., Houston, TX) sealed at the end with epoxy cement. To ensure each probe was inserted to the correct depth inside the guide shaft, a 3 mm long 21-gauge collar was soldered onto the 26-gauge probe body to act as a depth-stop. A 10 cm length of PE20 tubing was attached to the probe body via a fluid swivel and served as the inlet line for delivery of the dialysis perfusion medium. The silica glass tubing served as the outlet and was passed inside the PE20 tube through a small slit that was sealed with epoxy cement (Elmers Products, Inc., Columbus, OH). The silica outlet tube ran the length of the probe body and inside of the 0.75 mm dialysis fiber tip.

Microdialysis probes were implanted at least 16 hours prior to testing and were perfused overnight with a buffered Ringer's solution (142 mM NaCl, 3.9 mM KCl, 1.2 mM CaCl_2 , 1.0 mM MgCl_2 , 1.35 mM Na_2HPO_4 , 0.3 mM NaH_2PO_4 , 5.3 mM glucose, 0.3 μM neostigmine-Br; pH 7.3) at a flow rate of 0.5 $\mu\text{l}/\text{min}$. This time allowed the tissue trauma to dissipate and mice to habituate to the testing chamber. The following morning the flow rate was increased and maintained at 1.0 $\mu\text{l}/\text{min}$ during testing. Food and water were removed prior to testing.

Baseline samples were taken every 10 minutes for at least 60 minutes. For each animal, samples were alternatively analyzed using high-performance liquid chromatography (HPLC) for ACh and DA, resulting in a 20-minute sample interval for each neurotransmitter. Samples to be analyzed for DA were collected into 2 μl of 0.1M HCl / 0.1mM EDTA to prevent oxidation. Following baseline sampling, mice received either a vehicle perfusion test or were given a saline injection. The vehicle perfusion test consisted of intercalating a 20 cm length of PE20 tubing, preloaded with the Ringer's solution, into the input line of the microdialysis probe. Saline was injected IP in a volume of 0.1 ml per 25 grams. Samples were collected every 10 minutes over the next 80 minutes and alternately analyzed for ACh and DA content. Over the next 6 hours, each animal was given a systemic injection and an intra-VTA perfusion of methamphetamine. Separate groups of mice received the high doses (5 mg/kg IP and 1 mM intra-VTA) or low doses (2 mg/kg systemic and 100 μM intra-VTA) of methamphetamine. Order of treatment with respect to the route of administration was counterbalanced within the high and low dose categories.

Experiment 1: Measurement of VTA ACh and DA levels in response to intra-VTA perfusions of methamphetamine

Three 10 min samples collected for each neurotransmitter (60 minutes total) preceding the intra-VTA methamphetamine pulse served as baseline. A 20 cm length of PE20 tubing, preloaded with either 1 mM or 100 μM methamphetamine dissolved in the dialysis perfusion medium (20 μl volume), was intercalated into the input line of the microdialysis probe. Because the dialysis fiber likely allows only 10% of amines similar to methamphetamine to pass into tissue (based on *in vitro* tests with perfusion of dopamine), the perfusion lines were preloaded with relatively large concentration of methamphetamine. The Samples were taken every 10 minutes for 80 – 100 minutes and alternatively measured for ACh and DA.

Experiment 2: Measurement of VTA ACh and DA levels in response to systemic injections of methamphetamine

Three 10 min samples collected for each neurotransmitter (60 minutes total) preceding the IP injection of methamphetamine served as baseline. Animals were injected with 5 mg/kg or 2 mg/kg methamphetamine in a volume of 0.1 ml per 25 grams. Samples were taken every 10 minutes for 100 – 300 minutes and alternatively measured for ACh and DA.

Experiment 3: Effect of systemic methamphetamine on locomotor activity

Apparatus—Locomotor activity data were collected using Accuscan (Columbus, OH) photocell beam activity monitors (40×40×30 cm). Each monitor is housed within an acrylic chamber with sound attenuating foam lining the inside. In addition, a fluorescent light mounted at the top of the rear wall provides illumination and a fan, also located within each chamber, provides ventilation and ambient noise to aid in the masking of outside noises. Eight photocells are located 2 cm above the floor and record beam breaks as the animal moves about the chamber. These beam breaks are translated into distance traveled (cm). The bottom of the chamber was covered in a thin layer of corn cob bedding to absorb urine during the lengthy testing sessions and mimic the environment during experiment's 1 and 2. Animals were tested in the same order and at the same time of day and in the same monitor on days 1, 2 and 3. All behavioral testing occurred between 0900h and 1900h.

Procedure—Twenty-four mice were evaluated during two separate passes, for a total of twelve mice per pass and eight mice per dose (saline, 2 mg/kg methamphetamine and 5 mg/kg methamphetamine). Each pass was counterbalanced for methamphetamine dose and across chambers. The first pass began at 0900h and the second pass began at 1400h. This was done to mimic the time of day of injections during the first two experiments and to ensure no effect of time of day on methamphetamine-induced locomotor activation. There was no difference in the locomotor response between the two passes (data not shown). On Day 1 mice were moved in their home cages into the testing room 45–60 minutes before testing began in order to acclimate to the noises and room environment. Mice were weighed and placed into individual holding cages within 10 minutes being IP injected with 0.9% sterile saline and placed into the center of the activity monitor. Activity was recorded for 5 hours in 20 minute epochs. At the end of the 5 hour period, each animal was immediately removed from the chamber and placed back into its home cage. This procedure was repeated for all animals. Once all animals were evaluated, they were returned to their home room. On Day 2, mice were moved in their home cages into the testing room at the same time as they were on Day 1 and all procedures were repeated exactly described for Day 1. Day 1 testing served to provide habituation to handling and injection and provided a measure of locomotion in a novel environment, while Day 2 testing provided baseline activity data in habituated mice. On day 3 mice were handled exactly as on Days 1 and 2. For both passes of the study animals were injected with saline, 2 mg/kg methamphetamine or 5 mg/kg methamphetamine and placed directly into the activity chambers. Activity was monitored for 5 hours and when testing was complete, animals were euthanized by carbon dioxide asphyxiation.

Sample Analysis: High-Performance Liquid Chromatography (HPLC) Methods

Acetylcholine—Dialysates were measured for ACh by reverse phase HPLC with electrochemical detection. This system consisted of single piston pump and pulse dampener (SSI C., Fisher, St. Louis, MO), a 20 µl sample loop, and an amperometric detector (Waters Corp. Model 464; Milford, MA). Mobile phase for this system consisted of 200 mM potassium phosphate dibasic with 125 mg/L tetramethylammonium hydroxide. Monobasic potassium phosphate was used to adjust the pH to 7.8. ACh and choline were separated on an 8 cm, C18 analytical column (Varian Inc., Walnut Creek, CA) and then converted sequentially to betaine and hydrogen peroxide by an immobilized enzyme reactor (Varian). The resultant hydrogen peroxide was oxidized on a platinum electrode (BAS Inc., West Lafayette, IN) set at 0.5 V with respect to an Ag/AgCl reference electrode (Advanced Measurement Technologies, Oak Ridge, TN). Variability in sensitivity over time was measured by injecting aliquots of a known standard concentration at the beginning and end of each microdialysis session.

Dopamine—Dialysates were analyzed for DA by reverse phase HPLC with electrochemical detection (Epsilon amperometric detector; Bioanalytical Systems, West Lafayette, IN) with a

dual piston pump (BAS, Model PM80). DA was separated on a UniJet microbore column (BAS Inc, 1×100 mm, 3 μ m, ODS packing). Mobile phase for this system consisted of 100 mM monochloroacetic acid, 500 μ M EDTA, 600 μ M octanesulfonic acid and 8.6% v/v acetonitrile (pH 3.2). The mobile phase flow rate was 0.9 ml/min. Aliquots of known standard concentrations were injected into the HPLC system throughout the microdialysis session to provide a measure of variability in sensitivity over time.

Histology and Probe Verification

Once microdialysis sampling was complete, animals were overdosed with isoflurane. Brains were fixed with the probe in place in 10% formalin in PBS for two weeks and then placed in 20% sucrose, 10% formalin in PBS. Three days prior to sectioning, brains were transferred to 30% sucrose in PBS. Brains were sectioned on a Leica cryostat and 40 μ m sections were floated in a mounting solution (300 ml dH₂O, 1.5 g gelatin, 0.6 ml acetic acid, 200 ml 95% EtOH) prior to mounting onto slides. Slides were allowed to air dry before being thionin stained. A schematic of the probe locations was generated using histological drawings from the atlas of Paxinos and Franklin (Paxinos and Franklin, 2001) and is shown in Figure 1. For simplicity, probes placed in the left or right hemisphere are both shown in the figure, but each animal only received a unilateral implant.

Statistical Analyses

Microdialysis—Peak heights were measured for DA and ACh and then converted to mass (in moles) per 10 μ M and 4 μ M sample, respectively, by comparison to standard curves. Absolute levels of ACh and DA were not corrected for probe recovery because *in vitro* tests showed a less than 5% variation in recovery between probes made in our laboratory.

DA and ACh content in each sample was converted to a percentage of the mean of three baseline samples. Animals were counterbalanced for order of treatment (systemic injection versus intra-VTA pulse) and the samples were taken every 10 minutes and alternately measured for DA and ACh. We thus addressed two important concerns with the repeated measures design: a) whether the dialysate levels of ACh or DA were consistent when taken at 10 minutes or at 20 minutes and, b) whether the first methamphetamine treatment received (IP versus intra-VTA methamphetamine) affected the dialysate levels of ACh or DA following the second methamphetamine treatment. We grouped data by treatment and performed a two-way ANOVA to compare the DA and ACh levels from dialysates taken at 10 minutes or 20 minutes. No significant differences were found between 10-minute and 20-minute samples for DA or ACh, thus we collapsed across time creating 20-minute samples. To assess the potential effects of a prior methamphetamine treatment DA and ACh levels following subsequent methamphetamine treatments we compared, within each dose, the DA and ACh levels across time with treatment as the between-subjects variable. Two-way ANOVA indicated no significant differences in DA or ACh level whether an animal received the intra-VTA pulse first or following a systemic injection, or vice versa. Thus prior methamphetamine treatment had no significant effect on DA or ACh levels and all subsequent analyses were performed collapsed across treatment order (i.e. the two routes of administration). Separate two-way ANOVAs for systemic and locally applied methamphetamine were performed to assess the effect of drug dose (factor 1; 3 levels per route of administration) over time (factor 2; pseudo-repeated measures) on extrasynaptic DA and ACh levels. Since not every animal had a sample to analyze at each time point and some animals did not receive each drug dose per route of administration, the time factor was not a true repeated measure and the data were analyzed with factor 2 as non-matching. When significant interactions were obtained, simple main effects analyses were performed with Bonferroni post-hoc corrections. All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego,

CA, USA, www.graphpad.com). Data are expressed as the mean $\pm$ SEM and all results were considered significant if $P < 0.05$.

Locomotor Activity—The dependent variable used for analyses was horizontal distance traveled (centimeters). To determine an overall stimulatory effect of methamphetamine, a two-way repeated measure ANOVA was used to compare the total distance traveled between days 2 and 3. A separate two-way repeated measure ANOVA was performed on the data from day 2 and 3 to evaluate the effect of methamphetamine on locomotor activity across time. The 20-minute epochs were entered as the within subjects variable and drug as the between-subjects variable. Bonferroni corrected posttests were performed to further assess significant interactions. All analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com).

Results

Determination of baseline values and absolute levels of ACh and DA recovered in the VTA

Samples were taken during the animal's light cycle between 0900 and 1900 hours. Since the 5 mg/kg dose of methamphetamine caused robust activation, up to five hours of recovery samples were necessary for neurotransmitter levels to return to baseline and before making any further test. For all analyses, the reference basal level of ACh and DA was calculated by averaging the peak heights of the last three samples prior to drug treatment. During baseline measurements, the average absolute recovery of ACh and DA ($\pm$ SEM and not corrected for probe efficiency) across all subjects was 50.0 ± 3.0 fmol/10 μ M sample and 4.9 ± 0.07 fmol/4 μ M sample, respectively.

Experiment 1: Effects of intra-VTA methamphetamine on extrasynaptic ACh and DA in the VTA

Dopamine—Two-way ANOVA indicated that somatodendritic DA levels varied over time across drug condition as indicated by a significant drug $\times$ time interaction [$F(16,239) = 4.65$; $p < 0.0001$; Figure 2A]. Post-hoc analysis with Bonferroni correction indicated that the 1 mM methamphetamine treatment resulted in a significant 2473% increase above baseline in VTA DA levels compared to vehicle treatment during the 20-minute pulse perfusion ($t = 9.11$; $p < 0.001$). In contrast, perfusion of the Ringer's solution through the VTA did not significantly affect levels of extracellular DA (221% of baseline) during the pulse perfusion. Perfusion of 100 μ M methamphetamine was followed by a significant 1047% increase in baseline extracellular DA ($t = 3.27$; $p < 0.05$) during the 20-minute perfusion. Furthermore, DA levels during the 20-minute perfusion were significantly higher following 1mM than 100 μ M methamphetamine ($t = 5.77$; $p < 0.001$).

Acetylcholine—Neither dose of intra-VTA methamphetamine affected percent baseline ACh levels recovered across time [drug $\times$ time interaction: $F(16,234) = 0.37$; $p = 0.988$; Figure 2B]. However, there was a main effect of time [$F(8,234) = 2.59$; $p = 0.01$] with post-hoc analysis indicating that the time point when the pulse was intercalated into the perfusion line showed a significant increase in extrasynaptic ACh. The same magnitude increase in ACh occurred for all dose groups (including vehicle) during the 20-minute pulse perfusion and returned to baseline in the following sample. Although generally asleep during dialysis sampling, animals were often awakened when the methamphetamine pulses were intercalated into the perfusion line. We therefore attribute this generalized ACh increase to the disturbance induced by intercalating the pulse into the perfusion line.

Experiment 2: Effects of systemic methamphetamine on extrasynaptic ACh and DA in the VTA

Dopamine—Systemically administered methamphetamine resulted in a significant increase in VTA DA levels (main effect of drug: $F(2, 304)=7.00$; $p=0.001$) over time (main effect of time: $F(10,284)= 2.19$; $p=0.02$). Figure 3A shows that the 5 mg/kg dose of methamphetamine induced a significant increase in somatodendritic DA (941% above baseline, $t=4.41$, $p<0.001$) compared to saline treatment (177% above baseline). Administration of 2 mg/kg methamphetamine resulted in an increase in VTA DA (251% above baseline, $t=0.43$, $p>0.05$) that was not statistically different from saline injection.

Acetylcholine—Figure 3B shows the effect of IP injections of methamphetamine on percent baseline ACh recovered from the VTA. Two-way ANOVA indicated that extrasynaptic ACh varied significantly between drug conditions across time as indicated by a significant drug $\times$ time interaction [$F(20,348)= 5.12$; $p<0.0001$]. Post-hoc analysis indicated that the 5 mg/kg dose of methamphetamine resulted in significantly increased extracellular ACh levels (346% – 397% above baseline) compared to saline beginning at 20 minutes post-injection and continuing through 160 minutes post-injection (t -values ranged from $t=3.1$; $p<0.05$ to $t=7.03$; $p<0.001$). Injection of the 2 mg/kg dose of methamphetamine also increased extracellular ACh (221% – 275% above baseline), but was only significantly higher than saline-treated animals at 40 minutes post-injection ($t=3.87$; $p<0.01$). Saline injection resulted in a transient, 222% increase in ACh that returned to baseline by the next sample. Additionally, 5 mg/kg methamphetamine significantly increased extracellular ACh compared to 2 mg/kg methamphetamine at 20 minutes and 60 through 200 minutes post-injection (t -values ranged from $t=3.32$; $p<0.05$ to $t=5.6$; $p<0.001$). Interestingly, both doses of methamphetamine induced a prolonged increase in extracellular ACh, which took on average 180 minutes (for the 2 mg/kg dose) and 300 minutes (for the 5 mg/kg dose) to return to baseline (Figure 3B).

Experiment 3: Effects of systemic methamphetamine on locomotor activity

Two-way repeated measures ANOVA performed on total distance traveled within the five hour testing session indicated a significant day $\times$ drug interaction [$F(2,21)= 99.64$, $p<0.0001$; Figure 4A). Bonferroni posttests revealed that baseline locomotor activity on day 2 was not significantly different between the drug treatment groups (data not shown). On day 3 there was a significant increase in total distance traveled for mice that received 2 mg/kg methamphetamine ($t = 5.12$, $p<0.001$) or 5 mg/kg methamphetamine ($t = 14.06$, $p<0.001$) compared to saline-treated controls. Additionally, this relationship was dose dependent since mice that received 5 mg/kg methamphetamine were significantly more activated than mice that received 2 mg/kg methamphetamine ($t = 8.95$, $p<0.001$). Further evaluation of day 3 locomotor activity revealed a significant drug $\times$ epoch interaction [$F(28,294)= 16.30$, $p<0.0001$]; (Figure 4B). Mice that received 2 mg/kg methamphetamine were significantly more activated than saline controls for the first 80 minutes (t - values ranged $t=3.71$; $p<0.01$ to 8.58 ; $p< 0.001$). An injection of 5 mg/kg methamphetamine induced a significant increase in activity for the first 200 minutes compared to saline controls (t -values ranged from $t=3.24$; $p<0.05$ to 10.96 ; $p<0.001$). However, the dose-dependent relationship was more complex. The initial increase in locomotor activity dropped off quickly in the first hour but rose slightly between 80 and 120 minutes before continuing its decay to baseline. This created a more complex dose relationship between the two methamphetamine doses with the 5 mg/kg dose inducing significantly higher activity in the first 20 minutes ($t=7.21$, $p<0.01$) and from 80 – 180 minutes (t -values ranged from $t=4.47$; $p<0.001$ to $t=8.89$; $p<0.001$).

A Pearson's correlation analysis was performed to examine the relationship between locomotor activity and extracellular levels of ACh and DA across time. The correlation between activity and picograms ACh was strong following injections of 2 mg/kg methamphetamine ($R^2 = 0.70$,

$p=0.0003$) or 5 mg/kg methamphetamine ($R^2 = 0.85$, $p<0.0001$) (Figure 5A). In contrast, DA was not significantly correlated with methamphetamine-induced activity in a dose-dependent fashion. The lack of a dose-dependent correlation was most likely because 2 mg/kg methamphetamine was sub-threshold to increases extrasynaptic DA. Although 5 mg/kg methamphetamine yielded a significant correlation between extrasynaptic DA with locomotor activity ($R^2 = .47$, $p=0.005$), the relationship appeared not as strong across time compared to ACh (Figure 5B).

Discussion

A major finding of this study was the dissociation between the locus of methamphetamine effects on ACh levels in VTA compared to DA. Extracellular DA in the VTA was increased in a dose-dependent fashion by both systemic injection and focal infusion of methamphetamine into the VTA. In contrast, ACh in the VTA was increased only after a systemic methamphetamine challenge, and was not affected by intra-VTA methamphetamine infusion. These findings suggest that methamphetamine caused increased ACh output in VTA by stimulation of cholinergic activity outside of ACh terminals in the VTA.

The findings also demonstrate several components of the functional relationship between ACh and DA in VTA. We found that while ACh may participate in methamphetamine-induced DA increases in VTA (i.e. systemic methamphetamine increased ACh and DA), the methamphetamine-induced DA increase in the VTA is not dependent on ACh output (i.e. local perfusion of methamphetamine in the VTA increased DA output in the absence of a significant ACh response).

The time course of the increase in VTA ACh levels following systemic methamphetamine injection was much more prolonged compared to the large but transient increase in somatodendritic DA levels. Complementing the prolonged ACh increase was a protracted locomotor response following IP methamphetamine. Previous studies report prolonged locomotor activation following acute IP methamphetamine (Riviere et al., 1999) and amphetamine (Conti et al., 1997), but not after acute intra-VTA amphetamine (Kalivas and Weber, 1988, Vezina and Stewart, 1990). The initiation of the locomotor response was related to an increase in both ACh and DA, however only ACh appeared related to the protracted nature of the locomotor activation.

In contrast with systemic injection, intra-VTA perfusion of methamphetamine produced only a small and transient increase in extracellular ACh levels. However, the increase was similar in magnitude for methamphetamine and vehicle and returned to baseline immediately after the infusion. Thus, the change in ACh was non-selective for methamphetamine and suggests that disturbance caused by intercalating the pulse into the perfusion line resulted in a transient increase in VTA ACh.

Methamphetamine affects extracellular DA levels through several mechanisms

Several studies have performed microdialysis to measure DA output in terminal fields. For instance, extracellular DA increases in the NAc in response to natural rewards (Hernandez and Hoebel, 1988) and cholinergic agonists (Westerink et al., 1996, Gronier et al., 2000). The vast majority of drugs with abuse potential in humans cause increase DA transmission in animal models. Rats receiving passive or active systemic administration of cocaine (Bradberry and Roth, 1989), amphetamine (Ranaldi et al., 1999) and methamphetamine (Izawa et al., 2006) show an increase in extrasynaptic DA in the NAc. Within the NAc, methamphetamine is a potent substrate for the DA transporter DAT (for a review see Fleckenstein et al., 2007) and causes an increase in extracellular DA levels by reverse transport.

Several studies have performed microdialysis to evaluate mediators of somatodendritic DA release in the VTA of rats. Somatodendritic DA release is in part mediated by cholinergic muscarinic (Gronier and Rasmussen, 1998, Gronier et al., 2000) and nicotinic (Rahman et al., 2004) receptors. Other studies have focused on GABAergic and cholinergic feedback mechanisms that can mediate VTA somatodendritic DA release (Rahman and McBride, 2002) and how intra-VTA serotonergic tone affects somatodendritic DA release in different lines of rats (Liu et al., 2006). Additionally, several studies have found cocaine (Chen and Reith, 1994, Reith et al., 1997) and methamphetamine (Zhang et al., 2001) (Zhang et al., 2001) causes an increase in somatodendritic DA output in the rat VTA. However, to our knowledge there have been no published studies using microdialysis to assess the effects of methamphetamine on somatodendritic DA release in the VTA of mice.

Current evidence suggests methamphetamine acts on DA dendrites to affect somatodendritic release similarly to how it triggers DA release in terminal fields. Under normal conditions, the vesicular monoamine transporter-2 (VMAT2) transports cytoplasmic DA into reserpine-sensitive tubulovesicular organelles within the dendrites, thereby decreasing the cytoplasmic concentration of DA and preventing MAO metabolism (Nirenberg et al., 1996, Cooper, 2003). When the methamphetamine concentration is high, its lipophilicity facilitates diffusion into the dendrites via the plasmalemmal membrane causing a disruption of the VMAT2, thus preventing the sequestration of DA into vesicles (for reviews see Fleckenstein et al., 2007, Volz et al., 2007). The resulting build-up in cytoplasmic DA causes a reversal of the DAT on the dendrites and induces somatodendritic DA release.

Methamphetamine may also affect somatodendritic release through its ability to mediate DA cell firing. Methamphetamine induces calcium influx dependent oscillations in cytosolic calcium in VTA DA neurons (Uramura et al., 2000). These oscillations can directly influence DA neuron activation. However, the oscillations were observed following daily methamphetamine injections for 7 consecutive days. It is not known whether acute injection of methamphetamine, as used in this study, induces similar calcium flux. Nevertheless, our data show a rapid and strong increase in somatodendritic DA in response to methamphetamine treatment, which is consistent with stimulated release.

Following the increase, DA levels in VTA were rapidly decreased after methamphetamine injection. This quick return to baseline is consistent with other studies which found that, over time, psychostimulants including methamphetamine inhibit VTA DA firing presumably through activation of inhibitory D2 receptors following somatodendritic DA release (Kamata and Kameyama, 1985, Shi et al., 2004). The rapid return to baseline following intra-VTA methamphetamine may have been due to several factors. First, activation of D2 autoreceptors following somatodendritic DA release would cause an inhibition of DA cell firing (Aghajanian and Bunney, 1977, Grace and Bunney, 1985) which shuts down further release. However, since methamphetamine does not require depolarization to induce DA release and can independently modulate Ca^{2+} release, it is also possible that DA storage vesicles were depleted following such a robust response thereby disabling further DA release.

Intra-VTA dopamine and acetylcholine interactions

Excitatory cholinergic synapses on DA neurons may affect DA release in terminal fields (Gronier et al., 2000). Muscarinic (Lacey et al., 1990) and nicotinic (Calabresi et al., 1989) receptors in the VTA have been suggested to play a role in controlling VTA DA cell firing and both receptors have been implicated in mediating reinforcement (Yeomans and Baptista, 1997). Nicotine increases A10 DA neuron firing and this increase is blocked by mecamylamine (Mereu et al., 1987). Likewise, muscarinic ACh agonists induce increases in VTA DA release, which is prevented by pretreatment of the muscarinic antagonist n-methylscopolamine (Gronier et al., 2000). Moreover, stimulation of pontine cholinergic neurons causes an increase

in DA output in the NAc (Forster and Blaha, 2000). The DA response consists of a rapid phase followed by a slow decaying response that is dependent on M5 muscarinic receptors (Yeomans et al., 2001).

Morphology also suggests that ACh neurons could affect DA output. Cholinergic afferents form asymmetric synapses with VTA dopaminergic dendrites (Garzon et al., 1999, Omelchenko and Sesack, 2006). However, the time course of the increase in ACh levels was more prolonged compared to the large, transient increase in somatodendritic DA levels. These findings show that there is not a simple interaction between cholinergic and dopaminergic activity. While ACh may contribute to the activation of DA neurons (as described above), secondary mechanisms such as DA autoreceptor activation may act to oppose cholinergic stimulation and rectify DA levels in the VTA.

Methamphetamine acts on cholinergic neurons to affect VTA acetylcholine release

Since the ACh response only occurred when methamphetamine was administered systemically, we conclude that methamphetamine did not act directly on ACh terminal in the VTA to cause an increase in ACh levels. Cholinergic innervation of the VTA and SN from the lateral dorsal tegmental and pedunculopontine nuclei in the brainstem have been previously defined (Oakman et al., 1995, Bardo, 1998, Yeomans et al., 2001) and these afferents label with vesicular acetylcholine transporter (VAChT) indicating these terminals contain vesicles that release ACh (Garzon et al., 1999). Our data suggest that methamphetamine directly or indirectly activates these cholinergic projections to the VTA. The prolonged ACh response to systemic methamphetamine supports the idea that the cholinergic neurons sending afferents to the VTA do not habituate, but rather respond repeatedly after acute methamphetamine stimulation.

It is presently unknown which neural substrate(s) methamphetamine acts upon to cause the prolonged increase in VTA ACh output. Afferent connections to the pontine cholinergic cell bodies have been defined using retrograde and anterograde axonal transport methods. Projections have been described from the medial PFC (mPFC) and habenula to the LDTg. The bed nucleus of the stria terminalis (BNST) and the central nucleus of the amygdala send projections to the PPTg (Satoh and Fibiger, 1986, Semba and Fibiger, 1992, Steininger et al., 1992). Thus, the majority of afferents to pontine cholinergic and glutamatergic (Clements and Grant, 1990) cell groups come from limbic structures that are innervated by DA. Methamphetamine may thus activate a recurrent loop by initiating a cholinergic-mediated stimulation of mesocorticolimbic dopaminergic pathways (see also Forster and Blaha, 2000). Cortical and sub-cortical DA terminal fields send recurrent projections that activate the pontine nuclei, which in turn promote ACh release in VTA to propagate the signal. This suggests a functional pathway upon which methamphetamine could act to stimulate the mesolimbic system.

Methamphetamine-induced locomotor activity is associated with VTA dopamine and acetylcholine

In the current study, the relationship between the methamphetamine-induced locomotor activity and extrasynaptic ACh and DA is consistent with the idea that activation of dopaminergic fibers within the mesolimbic pathway is required for psychostimulant locomotor activation (Wise and Bozarth, 1987). Previous studies have found that methamphetamine and cocaine induce acute locomotion and increases in NAc DA levels in rats (Camp et al., 1994). Additionally, ongoing DA receptor activation is required for post-stereotypy locomotion after acute IP amphetamine (Conti et al., 1997) and the D1 DA receptor is necessary for cocaine-induced locomotor activation (Karasinska et al., 2005) and the activating effects of amphetamine (Xu et al., 2000). The DA-dependent nature of psychostimulant-induced locomotor activation suggests that other neurotransmitters, such as ACh, which alter the

dopaminergic tone, may also affect psychostimulant-induced locomotor activation. While M1 receptor knockouts exhibit exaggerated amphetamine-induced locomotion and extracellular DA levels within the NAc (Gerber et al., 2001), M5 receptor knockouts have only slight increases in scopolamine-induced locomotion (Chintoh et al., 2003), suggesting M5 receptors inhibit locomotor activation. However, M5 receptor knockouts specifically within the VTA exhibit no change in spontaneous, amphetamine- or scopolamine-induced locomotion (Yeomans et al., 2001). Thus, within the VTA a non-muscarinic mechanism is likely influencing the acute psychostimulant-induced locomotor activity.

The nicotinic receptor within the VTA is necessary for the nicotine-induced forward locomotion (Panagis et al., 1996, Alderson et al., 2005). Although it is well established that nicotinic ACh receptors in the VTA are required for the locomotor activating effects of nicotine, it is less clear that nicotinic mechanisms are responsible for methamphetamine-induced locomotor activity. Previous studies have failed to block the acute stimulating effects of methamphetamine and amphetamine with IP administration of subunit non-selective (mecamylamine) and α -7 selective (methylllycaconitine) nicotinic antagonists (Escubedo et al., 2005, Kamens and Phillips, 2008).

In addition to ACh, other neurotransmitters are also thought to be involved in acute psychostimulant activation. For instance, in the VTA inhibition of *tonic* Glu release results in an exaggeration of spontaneous and amphetamine-induced activation (Dalia et al., 1996).

The current study established that methamphetamine induces a prolonged pattern of locomotor activation, which more closely matches the increase in extrasynaptic ACh than DA within the VTA. Although, the current literature suggests that within the VTA ACh is more involved in mediating the motivational aspects of psychostimulants (Yeomans and Baptista, 1997, Yeomans et al., 2001, Ranaldi and Woolverton, 2002, Fink-Jensen et al., 2003, Carrigan and Dykstra, 2007a), our correlations suggest that while both DA and ACh may be important for the initiation of locomotor activation ACh alone appears to be involved in the *pattern* of the behavioral response. In conclusion we propose that methamphetamine activates a recurrent loop within the mesocorticolimbic pathway to simulate increases in VTA ACh and DA. Further studies will be needed to determine the behavioral sequelae of this form of recurrent activation.

Abbreviations

VTA, ventral tegmental area; NAc, nucleus accumbens; ACh, acetylcholine; DA, dopamine; DAT, dopamine transporter; mPFC, medial prefrontal cortex; LDTg, laterodorsal tegmental nucleus; PPTg, pedunculopontine tegmental nucleus; PBS, phosphate buffered saline; SN, substantia nigra.

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Figure 1.

Schematic representation of microdialysis probe placements in the ventral tegmental area (VTA) shown on histological drawings taken from the atlas of Paxinos and Franklin (2001). (A) Black bars indicate the location of each probe and the size of the 0.75 mm active dialysis fiber tip. Bars with green spheres represent animals that received the low dose of methamphetamine (2 mg/kg and/or 100 μ M) and bars with red triangles represent animals that received the high dose of methamphetamine (5 mg/kg and/or 1 mM). The numbers on the left-hand side of each coronal section indicate millimeters posterior from bregma. Orienting landmarks are labeled as follows: CA3 (CA3 region of the hippocampus), CA1 (CA1 region of the hippocampus), DG (dentate gyrus), ml (medial lemniscus), SNR (substantia nigra reticulata), SNC (substantia nigra pars compacta), fr (fasciculus retroflexus), 3V (3rd ventricle), LV (lateral ventricle). (B) Photograph of a thionin stained coronal brain slice demonstrating the track of the microdialysis probe. Red arrowheads represent the dorsal and ventral limits of the microdialysis fiber.

Figure 2.

Extracellular dopamine (A) and acetylcholine (B) levels in response to local applications of methamphetamine at 1 mM (red circles), 100 μ M (blue triangles) or vehicle (open squares) in the ventral tegmental area. The grey hatched bar represents the duration of time that methamphetamine or vehicle was perfused into the VTA by reverse dialysis. (A) Methamphetamine caused a dose-dependent increase in extracellular DA levels in the VTA during the 20 minute intra-VTA infusion, immediately after which DA levels returned to baseline. (B) Methamphetamine infusion had no significant effect on extracellular levels of ACh in the VTA. Time points marked with a single or double *asterisk* indicate a significant difference in extracellular DA levels between methamphetamine and vehicle treatment; * $p <$

0.05: 100 μ M versus vehicle; $**p < 0.01$: 1 mM versus vehicle and 100 μ M (two-way ANOVA with Bonferroni correction). Data are expressed as the mean ($\pm$ SEM) percent of baseline, which is indicated by the dotted line. Sample sizes are in parentheses beside each dose.

Figure 3.

Extracellular dopamine (A) and acetylcholine (B) levels measured from the ventral tegmental area in response to IP injections of methamphetamine at 5 mg/kg (red circles), 2 mg/kg (blue triangles) or saline (open squares). The time of the IP injection of methamphetamine or saline is marked with an arrow. (A) The 5 mg/kg dose of methamphetamine induced a robust increase in extracellular DA levels in the VTA, which returned to baseline shortly afterwards. Two-way ANOVA indicated a main effect of drug treatment ($*p < 0.001$). (B) IP injection of methamphetamine induced a dose-dependent and prolonged increase in extracellular ACh. $*P < 0.05$ versus saline and 2 mg/kg; $**P < 0.01$ versus saline; $***P < 0.01$ versus saline and 2 mg/kg. Treatment with 2 mg/kg and 5 mg/kg methamphetamine induced an increase in

extracellular ACh that took 180 and 300 minutes post-injection to return to baseline, respectively.

A

Figure 4.

Horizontal distance traveled (in centimeters) was measured for five hours in 20-minute epochs.

(A) To assess the acute activating effects of methamphetamine compared to baseline, total distance traveled was compared between Day 2 and 3. Baseline locomotor activity was measured for five hours on Day 2 following an IP injection of saline. Two-way ANOVA did not indicate any significant differences between treatment groups on total baseline activity. On Day 3, IP injection of 2 mg/kg methamphetamine (red circles) or 5 mg/kg methamphetamine (blue circles) induced a dose-dependent increase in total locomotor activity compared to saline-treated animals (white circles) (two-way ANOVA with Bonferroni correction). ** $P < 0.01$ versus saline; *** $P < 0.01$ versus saline and 2 mg/kg. (B) The pattern of methamphetamine activation on Day 3 was dose-dependent and varied across time (significant drug $\times$ epoch interaction [$F(28,294) = 16.30, p < 0.0001$]). 2- and 5-mg/kg methamphetamine induced significant activation for the first 80 and 200 minutes, respectively compared to saline-treated controls. 5 mg/kg methamphetamine induced more activity compared to 2 mg/kg methamphetamine treated animals in the first 20 minutes and from 80 – 180 minutes. ** $P < 0.01$ versus saline; *** $P < 0.001$ versus saline.

Figure 5.

Associations between methamphetamine-induced locomotor activation with extrasynaptic VTA ACh (A) and DA (B). (A) IP injection of 2 mg/kg (red) or 5 mg/kg (blue) methamphetamine resulted in significant correlations between locomotor activity (triangles, dashed line) and picograms ACh (circles, solid line). $R^2 = 0.70$ (2mg/kg); $R^2 = 0.85$ (5 mg/kg). (B) Extrasynaptic DA was not significantly correlated with methamphetamine-induced activity in a dose-dependent fashion. IP injection of 2 mg/kg methamphetamine (red) appeared to be a sub-threshold in stimulating DA (circles, solid line) and was not associated with locomotor activity (triangles, dashed line). IP injection of 5 mg/kg methamphetamine (blue) yielded a significant correlation between locomotor activity (triangles, dashed line) and picograms DA (circles, solid line). $R^2 = .47$