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A SENSITIZING REGIMEN OF METHAMPHETAMINE CAUSES IMPAIRMENTS IN A NOVELTY PREFERENCE TASK OF OBJECT RECOGNITION

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ABBREVIATIONS: 5-HT, serotonin; DA, dopamine; DAT, dopamine transporter; HC, hippocampus; mAMPH, methamphetamine; OR, object recognition; pRh, perirhinal cortex; RTI, [^{125}I]RTI-55; SAL, saline; SERT, serotonin transporter; STM, short-term memory

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ABSTRACT

A neurotoxic regimen of methamphetamine impairs object recognition (OR) in rats. The present study investigated whether a neurotoxicity is a necessary component of methamphetamine's effect on OR. Animals were exposed to a sensitizing regimen of methamphetamine, and were tested for OR one week, and locomotor behavior two weeks, later. Quantitative autoradiography was used to measure [^{125}I]RTI-55 binding to forebrain dopaminergic and serotonergic transporters. Methamphetamine treatment produced significant OR impairments (and increased locomotion), without reducing dopamine or serotonin transporter binding. This study supports the conclusion that factors other than monoamine terminal injury contribute to the methamphetamine-induced cognitive impairments.

Methamphetamine (mAMPH) is a widely-abused synthetic psychostimulant that produces a broad spectrum of behavioral effects. The drug's strong reinforcing qualities, its long-lasting effects, as well as the ease with which it can be manufactured has produced an epidemic of illicit drug use that has continued unabated.

In sufficient doses, exposure to mAMPH produces long-lasting damage to the dopaminergic [18, 38] and serotonergic systems [1, 32] as well as to cells in somatosensory cortex [14, 29]. The injury to monoaminergic terminals and to non-monoaminergic cell bodies has been most extensively reported in animals, but convergent evidence suggests that humans may also be subject to the neurotoxic effects of mAMPH [24, 46, 47; however, see 49].

A growing literature suggests that in addition to the neurochemical and structural alterations, mAMPH may result in long-term cognitive deficits. Clinically, current and abstinent mAMPH users show impairments in tests of verbal memory, manipulation of information, and motor performance [39, 42, 47]. Animals previously exposed to single-day neurotoxic mAMPH dosing regimens show motor performance deficits [48], impairments in an appetitive maze sequential learning task [12], and mild reductions in spatial memory acquisition [21]. More recently, several reports [4, 5, 37] have shown that rats exposed to neurotoxic doses of mAMPH are impaired in a task of novel object recognition (OR). Yet it is not clear whether the neurotoxicity observed after mAMPH treatment is necessary or sufficient to explain the memory impairment. Few studies have investigated non-neurotoxic stimulant-induced cognitive changes. Animals exposed to low, chronic doses of *d*-amphetamine show impaired object recognition [6] and lessened spontaneous alternation in a radial arm working memory task [11]. However, Stefani and

Moghaddam [41] did not find differences between rats pretreated with amphetamine or SAL in a T-Maze alternation task.

In order to explore more clearly whether mAMPH affects recognition memory independent of its potential to damage monoaminergic pathways, we employed a sensitizing dosing regimen of mAMPH. Chronic daily (or every other day) dosing regimens of mAMPH have been shown to induce a persistent heightened behavioral response to subsequent mAMPH administrations [27]. However, sensitizing dosing regimens of mAMPH or amphetamine do not produce long-term depletions of forebrain monoamine terminals [17, 23, 27].

Adult male Sprague-Dawley rats (275-300 g) were obtained from Charles River Laboratories (Hollister, CA) and individually housed, with food and water *ad libitum*, under a standard 12hr-light/12hr-dark cycle (lights on 7.00 – 19.00 hr) at a temperature of 22^{o}C. The protocol for this research was approved by the Institutional Animal Care and Use Committee of the University of California, Irvine. Acquisition, maintenance, handling, procedures, and care of the animals were in accord with the NIH Guide for the Care and Use of Laboratory Animals (NIH Guide, vol. 25, no. 28, 1996). Rats were treated every two days with a single injection of (+)methamphetamine hydrochloride (mAMPH; 3 mg/kg, i.p., Sigma, St. Louis, MO) or 0.9% sterile saline (SAL) for a total of 10 injections. All drug and vehicle injections were administered at a volume of 1 ml/kg, and doses are expressed as the free base. I.P. administration was used to conform to the procedures of [6]. All injections were given in the home cage.

One week after SAL or drug treatment the animals were exposed to a novelty preference task of object recognition. The object recognition task required that the rats

recall which of two small objects they had previously been exposed to. This task, widely used in the literature as a behavioral assay for recognition memory [19, 20, 26, 50], capitalizes on the fact that rats will explore novelty in a familiar environment. Thus, preferential exploration of an object is regarded as recognition memory. The task took place in a Plexiglas open field (40 x 40 x 38 cm high), the outside walls of which were covered with contact paper. A 15 W lamp placed 30 cm above the apparatus provided the only illumination in the room. The Familiarization phase was conducted by placing individual rats for 3 minutes into the field, in which two identical objects (objects A_1 and A_2) were positioned in two adjacent corners, 10 cm from the walls (Familiarization phase). In a short-term memory (STM) test given 90 minutes after familiarization, the rats explored the open field for 3 minutes in the presence of one familiar (A) and one novel (B) object. Objects were made of glass, plastic and metal and were chosen after determining, in preliminary experiments with other animals, that they were equally preferred. Between each trial both the open-field arena and the objects were washed with 95% ethanol solution. All sessions were videotaped, and an experimenter blind to treatment condition analyzed the OR behavior. Exploration was defined as sniffing or touching the object with the nose; sitting on the object was not considered exploration. Object placement was counterbalanced so that half of the animals in each treatment group saw the novel object on the left side (relative to the animal's starting position) of the open-field arena, and the other half saw the novel object on the right side of the arena. The proportion of the total exploration time that the animal spent investigating the novel object was the index of recognition memory. An exploration quotient (EQ) calculated for each animal was expressed by the ratio $T_B/(T_A+T_B)$. [T_A = time spent exploring the object

A; T_B = time spent exploring the object B]. The same formula was applied in order to get an EQ for the Familiarization phase. Assuming similar preferences for A1 and A2 during the Familiarization phase, EQ values for this phase approximate 0.5.

Two weeks following the last injection of mAMPH or saline, all animals were given a challenge dose of 1 mg/kg mAMPH (i.p.) and were tested for locomotor behavior. Testing began fifteen minutes after injections, and continued for eighteen minutes thereafter. Locomotor behavior was observed in opaque Plexiglas open fields (40 x 40 x 38 cm high), the bottoms of which were divided into four quadrants of equal size (delineated by colored tape). A crossing was counted when all four paws of an animal stepped over the boundaries of one quadrant into another. Sessions were recorded for later analysis by an observer blind to treatment conditions.

Twenty-four hours after completion of the last behavioral test, the rats were sacrificed, and their brains were removed and frozen at –20°C for use in autoradiography. Twenty μm-thick coronal sections were cut in a cryostat at the levels of the striatum and dorsal hippocampus, and sections were incubated with 21 pM [^{125}I]RTI-55 for autoradiographic localization of dopamine transporters (DAT) in the striatum and serotonin transporter (SERT) in the hippocampus and perirhinal cortex, using the procedures of [7]. DAT binding in these limbic areas (HC and pRh cortex) is quite low, and constitutes a small percentage of total binding [16, 33, 43]. Similarly, serotonin innervation constitutes only about 20% of the dopaminergic input to the striatum [10]. For these reasons, only DAT in striatum and SERT in HC and pRh were assessed. For striatal sections, DAT binding was defined as the total amount of [^{125}I]RTI-55 binding in the presence of the SERT inhibitor, fluoxetine (100 nM). For HC and pRh sections,

SERT binding was defined as the total amount of [^{125}I]RTI-55 binding. Slides containing tissue sections and standard slides containing known amounts of radioactivity were apposed to RayMax β autoradiography film (ICN Pharmaceuticals) for 48 hrs before development. Quantification of [^{125}I]RTI-55 binding to DAT and SERT in the autoradiographs was done on an MCID image analyzer (Imaging Research, St. Catherines, Ontario). Image densities were converted to [^{125}I]RTI-55 binding levels using a calibration curve based on readings taken from images of the standard slides packed with each film. Hippocampal and pRh SERT and striatal DAT levels were determined by outlining these structures (based on [31]) on their respective [^{125}I]RTI-55 images. The regions were quantified in the left and right hemispheres, and readings were averaged across hemispheres for at least three sections per animal. The HC and pRh were chosen because of their known involvement in tasks of learning and memory, including object recognition (see reviews by [9, 40]).

Exploration quotients (EQ scores) were analyzed using repeated measures analysis of variance (ANOVA). [^{125}I]RTI-55 binding results were analyzed using multivariate ANOVAs. Subsequent analyses of groups' performance on either the Familiarization phase or STM phase alone were conducted using independent samples Student's *t*-tests or one-way ANOVAs. Comparisons of EQ scores between familiarization and test sessions within the same group were done with paired-samples *t*-tests. Locomotor activity was scored as the total number of crossings during 1-6 minutes, 7-12 minutes, and 13-18 minutes, and the values from these 3 intervals were summed to yield the total crossings during the entire 18-min testing session. Repeated measures ANOVA was used to determine group differences in crossings within each of the three 6-minute time bins, and

Student's *t*-test was used to determine group differences during the 18 minutes. Group comparisons were done using one-tailed tests of significance with the *a priori* expectation that mAMPH-treated animals would not have EQ scores or [^{125}I]RTI-55 binding values higher than, or locomotor behavior scores lower than, saline-treated controls [4, 5, 37]. *P* values less than 0.05 were considered to indicate statistical significance.

Animals exposed to either SAL or 3 mg/kg mAMPH once every other day for 20 days showed no differences one week later in the Familiarization phase of the OR task, with both groups displaying equivalent overall amounts of object exploration ($A_1 + A_2$) [Independent-samples *t*-test, t=0.96, p=0.174]. Additionally, both groups explored the two copies of Object A equally [Paired samples *t*-tests, t=1.59, p=0.132 mAMPH; t=0.321, p=0.753 SAL]. These findings indicate that the sensitizing dosing regimen of mAMPH did not influence initial exploratory behavior and that the copies of the object in the left and right positions were equally preferred.

A repeated measures ANOVA revealed no significant main effect of treatment group [$F_{(1,30)}$=0.44, p>0.05], but a significant main effect of test phase [$F_{(1,30)}$=31.42, p<0.001] and a significant group by phase interaction [$F_{(1,30)}$=5.84, p=0.02; Fig. 1]. Additionally, an analysis of the performances at the STM test alone revealed a significant difference between the two groups [STM phase EQ score, Independent samples *t*-test, t=1.87, p=0.036]. Animals in both groups showed significant memory for the familiar object as evidenced by higher EQ scores during the STM compared to the Familiarization phase of the task [Familiarization phase EQ vs. STM EQ, paired samples *t*-test, t=2.37, p=0.031 mAMPH, and t=5.34, p<0.001 SAL; Fig. 1]. So although both groups showed significant preference for the novel object, SAL-treated control animals showed a stronger

preference than did mAMPH-treated animals. These data suggest that a sensitizing regimen of mAMPH impaired, but did not abolish, memory in the OR paradigm.

Repeated measures ANOVA revealed a group difference in locomotor activity across the three 6-minute locomotion intervals [$F_{(1,28)}$=4.61, p=0.02]. Additionally, animals exposed to the mAMPH dosing regimen showed greater overall locomotor activity during the 18 minutes of behavioral testing (relative to SAL controls) [Student's t-test, t=2.10, p=.023]. These results indicate that prior treatment with 3 mg/kg mAMPH (administered every other day for 20 days) caused a heightened response to a challenge dose of mAMPH, even after several days of abstinence (Fig. 2).

Animals treated with a sensitizing mAMPH dosing regimen had values for [^{125}I]RTI-55 binding to caudate-putamen and accumbens DAT as well as hippocampal and perirhinal SERT that did not differ from those of animals treated with SAL [Multivariate ANOVA, p>0.05 for group (Fig. 3,4)].

After rats were given a sensitizing mAMPH dosing regimen (3 mg/kg every other day for 20 days), they showed significant memory for the familiar object during the OR STM phase. However, these animals were impaired relative to SAL-treated control animals. Additionally, the mAMPH-treated animals in the present study showed evidence of locomotor sensitization one week after completion of the OR testing. These findings agree with those of Bisagno et al [6], who reported OR impairments in rats following a sensitizing regimen of *amphetamine*. The significant effect on OR memory observed in the present experiment occurred despite the absence of group differences in DAT or SERT in any region analyzed, suggesting that mAMPH exposure can induce cognitive impairments in the absence of damage to forebrain monoaminergic nerve

terminals. Although the monoamine transporters are subject to post-translational modification and cellular trafficking [44], thereby complicating interpretation of radioligand binding to these transport sites, the conclusion that sensitizing daily dosing regimens of amphetamines do not damage forebrain dopamine terminals is supported by other experiments utilizing other markers (e.g., striatal DA content) of terminal integrity [17, 23, 27].

Although the underlying cause of the OR impairment in mAMPH-sensitized rats remains uncertain, a role for amphetamine-induced changes in dendrites and dendritic spines in, e.g. the nucleus accumbens or prefrontal cortex [35] merits consideration. Repeated exposure to amphetamines in a sensitizing regimen interferes with the subsequent ability of a complex environment to increase dendritic branching and spine density within the nucleus accumbens and somatosensory cortex [22]. This amphetamine treatment also blocks the ability of housing in a complex environment to increase fear conditioning [8], raising the possibility that exposure to amphetamines, without consequent neurotoxicity, will blunt certain forms of new learning by occluding the brain's structural plasticity.

Additionally, a possible role of stress hormones warrants consideration. A role of the hypothalamo-pituitary-adrenocortical (HPA) axis in facilitating behavioral and neurochemical psychostimulant sensitization has been proposed. Amphetamine-induced sensitization is prevented by adrenalectomy [34] and also by administration of corticotropin-releasing hormone antibodies [13]. Animals previously exposed to sensitizing dosing regimens of amphetamines show a heightened neuroendocrine response either to challenge doses of amphetamines or to physical stressors [3, 15, 28, 36,

45]. It is known that either stress or administration of corticosterone (a glucocorticoid, levels of which are elevated in response to stressors) affects performance in the novelty-preference recognition memory task [2, 25, 30]. Thus, it may be that a heightened responsiveness of the HPA axis during the time of OR testing may have contributed to this group's OR impairment.

In conclusion, while the present results do not argue against a role of monoamine neurotoxicity in the cognitive impairments resulting from single-day, neurotoxic mAMPH regimens [4, 37], a sensitizing mAMPH dose regimen may affect memory via mechanisms distinct from neurotoxicity.

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FIGURE LEGENDS

Figure 1. Animals subjected to a sensitizing mAMPH dosing regimen (mAMPH-SENSITIZED) were moderately impaired relative to saline-treated controls when tested on the novelty preference test of object recognition one week later. Data expressed as mean $\pm$ SEM exploration quotients. Repeated measures ANOVA revealed a significant interaction between group and phase of OR testing [$F_{(1,30)}$=5.84, p=0.02]. *Indicates significant difference in exploration quotients of saline and mAMPH-sensitized groups during STM phase (Independent-samples *t*-test), $p<0.04$.

Figure 2. Animals treated with a sensitizing regimen of mAMPH had a significantly greater number of crossings than SAL-treated controls in a locomotor test given following challenge with 1 mg/kg mAMPH two weeks after withdrawal from mAMPH. Data for both graphs are expressed as mean $\pm$ SEM. Line graph depicts locomotor activity (crossings) for each of the three time periods analyzed (i.e., minutes 1-6, 7-12 and 13-18), and bar graph depicts crossings for the entire 18-minute period. Open figures indicate treatment with saline and black figures indicate treatment with sensitizing mAMPH. *Indicates significant difference from SAL-treated cohort (Independent-samples *t*-test) $p<0.03$.

Figure 3. RTI binding to dopaminergic transporters (DAT) in the dorsal (dCPu) and ventral caudate putamen (vCPu), and nucleus accumbens (NAc), and serotonergic transporters (SERT) in hippocampus (HC), and perirhinal cortex (pRh Cx). Values represent mean $\pm$ binding levels, expressed in μCi per gram of tissue.

Figure 4. Dopamine transporter (DAT) and serotonin transporter (SERT) binding sites in brains of animals treated with saline or a sensitizing mAMPH dosing regimen (mAMPH-SENSITIZED). Autoradiographic images of [^{125}I]RTI-55 binding to striatal DAT (top row), hippocampal SERT (middle row) and perirhinal SERT (arrow, bottom row) of rats used in the behavioral tasks two weeks after saline (**A,C,E)** or sensitizing mAMPH (**B,D,F**) injections.