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Research report

Expression of *c-fos* mRNA in the basal ganglia associated with contingent tolerance to amphetamine-induced hypophagia

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ABSTRACT

Tolerance to the hypophagic effect of psychostimulants is contingent on having access to food while intoxicated. Rats given chronic injections of such drugs with access to food learn to suppress stereotyped movements, which interfere with feeding. In contrast, controls given the drug after food access do not learn to suppress stereotypy and, therefore, do not become tolerant. To determine the role of the basal ganglia in this phenomenon, we used *in situ* hybridization to measure the expression of *c-fos* mRNA, a marker for neural activation, in the brains of tolerant and nontolerant rats. Rats given chronic amphetamine injections prior to food access learned to suppress stereotyped movements, whereas yoked controls given the drug after feeding did not. Following an acute injection of amphetamine, both of these groups had higher levels of *c-fos* mRNA than saline-treated controls throughout the striatum, in the nucleus accumbens core, the ventral pallidum and layers V–VI of the motor cortex. In contrast, tolerant rats, which had learned to suppress stereotypy, had higher levels of *c-fos* mRNA than both amphetamine- and saline-treated controls in the entopeduncular nucleus, globus pallidus, subthalamic nucleus, pedunculopontine nucleus, nucleus accumbens shell, olfactory tubercle, somatosensory cortex, and layers II–IV of motor cortex. These data suggest that the learned suppression of amphetamine-induced stereotypy involves the activation of dorsal striatal pathways previously implicated in response selection as well as the ventral striatum, long implicated in appetitive motivation and reinforcement.

1. Introduction

In addition to being potent drugs of abuse, psychostimulants induce locomotion and stereotyped movements and suppress food intake. There is increasing evidence that the environmental context in which these drugs are administered exerts a profound influence on the expression of these behavioral effects [6,50]. For example, the increase in locomotion induced by acute administration of amphetamine is greater when the drug is administered in a novel environment than when it is given in the home cage [7,8]. Similarly, sensitization of locomotion following chronic administration of psychostimulants is greater when the drug is administered in a novel environment [7,8].

Environmental context also plays a critical role in the development of tolerance to the hypophagic effects of psychostimulants [60]. In this case, tolerance is contingent on experiencing the drug in an environment containing food. Rats given psychostimulants and access to sweetened milk develop tolerance to drug-induced hypophagia, whereas rats given the same number of injections *after* they have had access to milk do not [12,16,25]. Such "contingent tolerance" is mediated by the learned suppression of stereotyped head movements, which interfere with feeding. When behavioral interference from stereotypy is bypassed by delivering milk directly into the mouth through intraoral cannulas, rats show little hypophagia to psychostimulants [61,64,66]. This demonstrates that food remains reinforcing and, therefore, can serve as an incentive for learning to suppress stereotypy. Indeed, amphetamine-treated rats learn to maintain a stationary head position when reinforced with intraoral infusions of milk and they do so at the same rate at which bottle-fed rats develop tolerance [65]. Remarkably, rats learn to suppress stereotyped movements even after sensitization develops [61–63].

Little is known about the neural mechanisms underlying contingent tolerance to amphetamine. Understanding the neural basis of this phenomenon would broaden our understanding of how environmental context modulates psychostimulant drug effects. Moreover, amphetamine-induced stereotypy in rodents is often used to model dyskinesias and tics in humans [14,25]. These involuntary movements can be at least partially suppressed by

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reinforcement-based behavioral therapies, such as "habit reversal" [4,45,58]. Thus, understanding the neural mechanisms underlying the learned suppression of stereotypy may have clinical relevance as well.

Previous research has established that stereotypy involves the dorsal striatum and its output pathways (see Section 4), whereas the reinforcing effects of both natural rewards and psychoactive drugs involve the ventral striatum and associated structures [33,34]. It seems likely, therefore, that the learned suppression of stereotyped movements involves changes in the neural activity of at least some of these brain regions. As a first approach to addressing this issue, we examined levels of mRNA expression for the immediate-early gene *c-fos*, used here as a marker for neural activation, in the basal ganglia of tolerant and nontolerant rats following acute injection of amphetamine. At doses that induce stereotyped movements, amphetamine increases *c-fos* expression in the basal ganglia [15,20,26] and both the degree and the pattern of *c-fos* expression are strongly influenced by the environmental context in which the drug is given. For example, the expression of *c-fos* in both the dorsal and ventral striatum is greater if amphetamine is given in a novel environment than if it is given in the home cage [9,55]. Moreover, whereas amphetamine induces *c-fos* primarily in D1/prodynorphin-expressing neurons when administered in the home cage, it induces *c-fos* in both D1/prodynorphin- and D2/proenkephalin-expressing neurons when it is administered in a novel environment [10,56]. Finally, environmental context influences the particular structures in which amphetamine induces *c-fos*. For example, levels of *c-fos* are increased in the subthalamic nucleus when amphetamine is given in a novel environment, but not when it is given in the home cage [56,57]. Based on these findings, we expected that tolerant rats, which had been given injections of amphetamine in an environment containing food, would display a different pattern of *c-fos* mRNA expression within the basal ganglia than nontolerant rats given the same number of amphetamine injections in the absence of food.

2. Materials and methods

2.1. Subjects

Subjects were 46 male albino Sprague–Dawley rats (Harlan, Indianapolis, IN) weighing 250–275 g at the beginning of the experiment. They were housed and tested in individual stainless steel cages (17.5 cm W × 25.0 cm L × 17.5 cm H) with wire mesh fronts and bottoms in a room maintained at 24°C under a 12 h light/dark cycle (lights on 0700 h). The rats were fed three Purina lab chow pellets (Ralston Purina Company, St. Louis; about 15 g) and unlimited water daily. On days in which tests were not conducted, the rats were given an extra food pellet (about 5 g). Rats generally consume about 30 g of chow daily when fed *ad libitum*. Therefore, the restricted availability of chow ensured that the rats were moderately food deprived during the initial stages of the experiment, prior to the development of tolerance. All procedures were approved by the university's Institutional Animal Care and Use Committee and conformed to NIH guidelines.

2.2. Drugs

D-Amphetamine sulfate (Sigma, St. Louis) was dissolved in sterile isotonic saline and injected in a volume of 1 ml/kg. Doses of the drug are expressed as the weight of the salt. Sodium pentobarbital (Abbott Laboratories, N. Chicago, IL) was administered at a dose of 150 mg/kg. All injections were intraperitoneal.

2.3. Milk tests

In the first phase of the experiment, the rats were given injections of saline (1 ml/kg) both before and after the milk tests for 36 trials to establish a stable baseline of milk intake. Milk tests were conducted 6 days per week, in the morning. On test days, water bottles were removed from the cages, the rats were weighed, injected with saline, and 20 min later given Eagle Brand sweetened condensed milk (Borden, Columbus, OH) diluted with water (1:3) in calibrated drinking tubes attached to the front of the cages for 30 min. At the end of the test, intakes were recorded and the drinking tubes were removed. Approximately 30 min later, the rats were given a second injection of saline. They were then fed and their water bottles were returned.

In the next phase of the experiment, dose–response tests were conducted to assess the rats' sensitivity to amphetamine. Test doses of amphetamine (0.5, 1, 2 and 4 mg/kg) and saline were administered in counterbalanced order 20 min prior to the milk tests with at least 3 days between doses. On the intervening days, saline injections were given. In addition to measuring milk intake, motor activity was rated seven times during each trial: 5 min before milk access, 5, 10, 15, 20 and 25 min after milk was presented, and 5 min after the milk bottles were removed. Motor activity was assessed using a 6-point nominal rating scale, which included the following categories: 0 = stationary and immobile; 1 = stationary activity without stereotyped head movements (e.g., grooming, drinking); 2 = movement involving one or both forelimbs without concurrent stereotyped head movements (e.g., pivoting, rearing, walking); 3 = stereotyped head movements accompanied by sniffing, and generally covering a wide area; 4 = focused stereotyped head scanning movements covering a small area of the wall or floor of the cage; and 5 = stereotyped mouthing, licking, biting, or gnawing. At each rating interval, each rat was observed for about 5 s by a trained observer, who scored the dominant behavior that occurred in that interval. Following the dose–response trials, the rats were given seven additional trials with both pre- and post-test injections of saline to re-establish the baseline.

Five rats that were insensitive to the hypophagic effect of amphetamine were eliminated from the experiment at this point. The remaining rats were rank-ordered based on their milk intakes under the 2 mg/kg dose of amphetamine. Cohorts of comparably sensitive rats were then randomly assigned to one of four groups, with the extra rat assigned to the Tolerant group (see below). During the tolerance phase of the experiment, rats in the Tolerant group were given injections of amphetamine (2 mg/kg) 20 min before the milk tests and injections of saline 30 min afterward. The dose of amphetamine was selected on the basis of previous studies on contingent tolerance to amphetamine. Rats in two pharmacological control groups (Amphet/After 1 and Amphet/After 2), were given the injections in the reverse order; i.e., saline before the milk tests and amphetamine afterward. Rats in the saline control group were given injections of saline both before and after the milk tests. In order to control for differences in milk intakes between the groups, the intakes of all three control groups were yoked to those of the Tolerant group. This was accomplished by staggering the trials by 1 day, so that the latter groups were given the mean amount of milk ingested by the Tolerant group on the previous day.

During the course of the tolerance phase, three additional rats were eliminated from the experiment. Two of these rats became extremely irritable when handled and the other inexplicably stopped drinking milk. Consequently, the final composition of the groups was as follows: Tolerant (10), Amphet/After 1 (8), Amphet/After 2 (10), and Saline (10).

For the purposes of this experiment, tolerance was defined as recovery of milk intake to at least 70% of baseline levels. When an individual rat in the Tolerant group reached this level of milk intake (mean: 35 days), both that rat and one rat from each of the control groups were given a final milk test on the following day, during which milk intake was unrestricted. On this final test, rats in both the Tolerant group and the Amphet/After 1 group were given injections of amphetamine 20 min before the test. Both of these groups had identical histories of amphetamine treatment and milk intake during the tolerance phase of the experiment, although the temporal relation between these events was different. Thus, the Amphet/After 1 group served as a nontolerant pharmacological control group. Rats in the Amphet/After 2 group and the Saline group were given pre-test injections of saline on the final test day. These groups controlled for the effects of having a history of amphetamine injections during the tolerance phase (Amphet/After 2 group) and for the effects of having a history of milk intake during the tolerance phase (Saline group). Thirty minutes after presentation of the milk on the final test day, the rats were euthanized with an injection of sodium pentobarbital and their brains were removed, frozen in isopentane, and stored at −70° C.

2.4. In situ hybridization

Coronal serial brain sections (one in seven; 20 μm) were cut in a cryostat at −20 °C, postfixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), collected onto charged glass slides, and stored at −20 °C. Tissue was processed for *in situ* hybridization using in vitro-transcribed ^{35}S-labeled RNA complementary to rat c-*fos* mRNA as previously described [27]. Antisense c-*fos* RNA comprised a 667 base transcript complementary to positions 583–1250 of the clone pc*fos*(rat)-1 ([21]; GenBank sequence X06769). Hybridization was conducted at 60 °C for 16–18 h with the cRNA at a final concentration of 1×10^7 cpm/ml. Posthybridization treatment included incubation with RNase A (12 Kunitz units/ml) for 30 min at 45 °C. All hybridizations included brain tissue from a cohort of matched animals representing all treatment groups. Labeling specificity was verified by hybridization of some sections with ^{35}S-labeled c-*fos* sense RNA. The distribution of c-*fos* cRNA hybridization was evaluated using Kodak Biomax MR film and Kodak NTB emulsion autoradiography with exposure intervals of 13 days and 13 weeks, respectively. Tissue was stained with cresyl violet or hematoxylin for microscopic analysis. Sections were viewed and photographed with an Olympus AX70 microscope equipped with a Magnafire digital camera. Composite images were prepared in Adobe Photoshop, with adjustments made for brightness and contrast.

2.5. Densitometry

Comparisons of neural activation patterns across animals were made using film autoradiography. Autoradiograms were digitized and analyzed on a computer-based