Brain Activity during Methamphetamine Anticipation in a Non-Invasive Self-Administration Paradigm in Mice

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Visual Abstract

The ability to sense time and anticipate events is critical for survival. Learned responses that allow anticipation of the availability of food or water have been intensively studied. While anticipatory behaviors also occur prior to availability of regularly available rewards, there has been relatively little work on anticipation of drugs of abuse, specifically methamphetamine (MA). In the present study, we used a protocol that avoided possible CNS effects of stresses of handling or surgery by testing anticipation of MA availability in animals living in their home cages, with daily voluntary access to the drug at a fixed time of day. Anticipation was operationalized as the amount of wheel running prior to MA availability. Mice were divided into four groups given access to either nebulized MA or water, in early or late day. Animals with access to MA, but not water controls, showed anticipatory activity, with more anticipation in early compared to late day and significant interaction effects.

Next, we explored the neural basis of the MA anticipation, using c-FOS expression, in animals eutha-
nized at the usual time of nebulization access. In the dorsomedial hypothalamus (DMH) and orbitofrontal cortex (OFC), the pattern of c-FOS expression paralleled that of anticipatory behavior, with significant main and interaction effects of treatment and time of day. The results for the lateral septum (LS) were significant for main effects and marginally significant for interaction effects. These studies suggest that anticipation of MA is associated with activation of brain regions important in circadian timing, emotional regulation, and decision making.

Key words: anticipation; circadian; dorsomedial hypothalamus; lateral septum; nebulization; orbitofrontal cortex

Introduction

Abuse of methamphetamine (MA) is an international public health problem with an estimated 15–16 million users worldwide, making MA the second most widely abused drug after cannabis (United-Nations, 2011). Abuse of a psychostimulant such as MA has adverse and widespread consequences for the central nervous system (Richards and Laurin, 2018). While consequences of MA intake administered in the drinking water (Tataroglu et al., 2006; Honma and Honma, 2009) have been amply examined, the neural responses associated with the anticipation of MA availability are less well understood. Anticipation and prediction are fundamental functions of the brain; signals that a reward is imminent are associated with not only MA and other drugs, but also with rewards such as alcohol, food, highly palatable rewards and sweets, and sex (Pitchers et al., 2013; Webb et al., 2015).

Such signals include distinctive external visual, auditory, or olfactory cues, and interoceptive responses. For example, before regularly scheduled meals, the CNS and peripheral organs produce signals that anticipate the availability of nutrients, thereby preparing the body for food intake [mouse (LeSauter et al., 2009), rat (Patton et al., 2014), and human (Ott et al., 2011, 2012); Patton and Mistlberger, 2013; Challet, 2015]. While MA anticipation has not been directly tested in humans, there is evidence of contextual preference for stimuli paired with MA administration (Childs and de Wit, 2009; 2013; Mayo et al., 2013).

The circadian timing system is an important component of anticipation of daily recurring future events (Mellers et al., 1999). Circadian timing occurs in the absence of all external timing signals, and is a function of the brain’s master clock in the suprachiasmatic nucleus. Numerous studies demonstrate that when food or a palatable treat reward are offered to ad libitum fed rats during their sleep time, animals will anticipate by awakening hours before the appearance of the food (Mistlberger, 1994; Escobar et al., 2011). This phenomenon is also seen in nature (Caba and González-Mariscal, 2009) and in response to rewards other than food (Webb et al., 2009), and can occur to multiple regularly timed events each day (Stephan, 1983; Mistlberger et al., 2012). Though there are many parallels between food and drug reward systems (Alonso-Alonso et al., 2015; Tomasi et al., 2015), anticipatory interoceptive cues have been little studied in the context of time-of-day effects on drug intake (Siegel and Ramos, 2002; Siegel, 2005). That said, there is evidence that activation of pleasant interoceptive signals is a component of addictive behaviors (Stewart et al., 2015).

There are circadian effects on behaviors associated with anticipation of regularly scheduled drug injections. Following daily injections of MA, there is a gradual elevation, during the animal’s normal sleep time, of locomotor activity in the time preceding the injection (Shibata et al., 1994). However, anticipatory activity does not appear in the absence of a circadian injection schedule (Iijima et al., 2002), indicating that entrainment of the circadian timing system is required for the anticipation to develop. More evidence of a circadian component to anticipation is available in changes in c-FOS expression in anticipation of a daily meal, with studies in rats (Challet et al., 1997; Angeles-Castellanos et al., 2004, 2007; Mendoza et al., 2005; Escobar et al., 2007; Poulin and Timofeeva, 2008; Acosta-Galvan et al., 2011; Mitra et al., 2011; Caba et al., 2014), mice (Begriche et al., 2012; Blum et al., 2012; Gallardo et al., 2014; Dattolo et al., 2016; Luna-Illades et al., 2017), and hamsters (Dantas-Ferreira et al., 2015; Ruby et al., 2017), or in anticipation of a palatable treat in rats or mice (Mendoza et al., 2005; Mitra et al., 2011; Gallardo et al., 2012; Blancas et al., 2014).

For studies of drugs of abuse, the gold standard entails self-administration. Here we are interested in anticipatory responses associated with voluntary intake of MA. We use a non-invasive protocol that eliminates possible CNS effects of stress associated with handling, injections or surgery that may alter the anticipatory response to the drug. In this protocol, mice live in their home cage and have regularly scheduled daily access to nebulized MA or water for 1h via a tunnel that leads to a chamber where the drug is nebulized and available during their normal sleep.
time (Juarez-Portilla et al., 2017). Here, we used this protocol to examine behavioral anticipation of MA availability and to identify c-FOS expression at the time of anticipation, before the availability of the drug.

The efficacy of the nebulized MA in this protocol has been previously demonstrated in several responses (Juarez-Portilla et al., 2017). Mice spend average of ~3 min in the chamber during the interval of MA availability. Elevated locomotor activity occurs during the 1 h of MA availability and for the 3 h thereafter. On the other hand, control mice with access to nebulized water have consistently low activity levels (Juarez-Portilla et al., 2017; Fig. 4). Importantly, following 3-min experimenter-imposed exposure to nebulized MA, serum levels are elevated in mice euthanized 20, 60, or 120 min later. Finally, the amount of time mice spend in the nebulizing chamber is inversely proportional to the concentration of nebulized MA indicating that they self-regulate their intake of MA (Juarez-Portilla et al., 2017; Fig. 5B).

Materials and Methods

Animals and housing

Male mice (strain C57BL/6N) were purchased from Jackson Laboratory at five to six weeks of age. The animals were group-housed (4 per cage, 28 × 17 × 12 cm) for 10 d on arrival and subsequently were housed individually in cages (32 × 14 × 13 cm) made of clear polycarbonate, provided with pine shavings and a running wheel (11 cm in diameter). The wheel had a magnetic sensor connected to a computer enabling continuous monitoring of wheel revolutions. Standard mouse chow (Lab-Diet 5001; PMI Nutrition) and water were provided ad libitum, and room temperature was maintained at 21 ± 1°C. A dim red light (<1 lux) was on at all times, allowing for animal handling and maintenance. Mice were housed for 17 d in a 12/12 h light/dark (LD) cycle, with lights on defined as zeitgeber time 0 (ZT0) and lights off as ZT12. Entrainment was confirmed for all animals. On experimental days, a skeleton photoperiod that allowed for continued entrainment was used, with lights on for 30 min at the beginning and end of the animal’s day. In skeleton photoperiods, animals continue their behavior as though it were a full photoperiod, with their inactive phase (subjective day) at the prior time of lights on, and their active phase (subjective night) at the prior time of lights off (Patton et al., 2013; Rosenwasser et al., 2015). This lighting regimen provides the advantage of avoiding “masking”; i.e., the direct suppressive effects of light on activity that occurs in nocturnal species (Pittendrigh and Minis, 1964; Pittendrigh, 1981; Mrosovsky and Hattar, 2003). All experimental procedures were approved and conducted according to the Author’s University Institutional Animal Care and Use Committee.

Test apparatus and protocol

The test apparatus, consisted of a tunnel (7.1 × 3.1 × 3.1 cm) connecting the home cage to a nebulization chamber (11.4 × 11.4 × 6 cm), as previously described (Juarez-Portilla et al., 2017). For delivery of vaporized material, a nebulizer (catalog #40-370-000; Briggs Medi-

Drug preparation

MA hydrochloride (catalog #M8750-5G, Sigma-Aldrich Corp) was dissolved in water at a concentration of 1 mg/ml and nebulized for experimental animals. For control animals, water was nebulized. A volume of 7.5 ml of nebulized MA or water was expressed from the nebulizer into the chamber during the 1-h interval of availability each day.

Experimental groups

Animals were divided into four groups (N = 6/group), as follows: water (W) or MA was nebulized in the chamber at either ZT4–ZT5 or at ZT10–ZT11 (group names, MA-ZT4, W-ZT4, MA-ZT10, and W-ZT10). One animal from the MA-ZT4 group failed to entrain to the skeleton photoperiod and was removed from the study.

Behavioral measures and analysis

Locomotor activity was quantified by monitoring wheel revolutions in 10-min bins throughout the 24-h day, using Vitalview Software (RRID:SCR_014497, Minimitter Inc.). Actograms were plotted using Clock Lab (RRID:SCR_014309, Actimetrics). To establish group means, data from each animal were normalized using standard methods (Landry et al., 2007; Butler and Silver, 2011; Gallardo et al., 2014). Thus, the amount of locomotor activity during the anticipatory interval, before MA availability, was assessed as a fraction of the individual’s total locomotor activity (Figs. 2C, 3A). The number of wheel revolutions per 10-min bin divided by the average daily rotations and multiplied by 144 (the number of 10-min bins/d). For analysis of anticipatory behavior, wheel
running activity was analyzed for the last 10 d of the experimental period. Data were not normalized for determination of total daily activity (Fig. 3B) or when records of individual animals are shown (Fig. 2B).

**Perfusion, tissue processing, and staining**

Mice were anaesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively; i.p.) at ZT4 or ZT10 and were perfused transcardially with 50 ml of saline solution (0.9%)
followed by 100 ml of 4% paraformaldehyde in phosphate buffer (PB, pH 7.3). Brains were removed, postfixed for 24 h and cryoprotected in 20% sucrose in PB and sectioned coronally at 50 μm in a cryostat (Microm HM 500M) at −20°C. For immunohistochemistry, serial coronal sections from the olfactory bulb (OB) to the posterior mid-brain were collected in PB, and processed free floating. Every fourth section was used for double-label fluorescence immunostaining of c-FOS and tyrosine hydroxylase (TH). Sections were incubated in blocking solution of normal donkey serum (catalog #017-000-121 RRID: AB_2337258; Jackson ImmunoResearch) in PB containing 0.3% of Triton X-100 (PBT 0.3%) for 1 h, then incubated for 48 h at 4°C in c-FOS antibody made in rabbit (1:5000; catalog #sc-52, RRID: AB_2106783; Santa Cruz Biotechnology) and a monoclonal TH antibody made in mouse (1:5000; catalog #2294, RRID: AB_572268, Immunostar) diluted in PBT 0.3%. Thereafter, sections were incubated in donkey secondary antibody conjugated to a Cy3 anti-rabbit (catalog #711-165-152, RRID: AB_2307443) and Cy2 anti-mouse (catalog #715-225-150, RRID:AB_2340826) fluorophores (1:200; Jackson ImmunoResearch) for 2 h, tissue was washed with PBT 0.1% between incubations. Finally, sections were mounted onto gelatin-subbed slides, dehydrated, cleared in CitriSolv (Fisher Scientific), and coverslipped with Krytalon (EM Diagnostics).

Tissue analysis
To select brain regions for cell counting of c-FOS cells, two strategies were implemented. First, all sections through the entire brain were scanned to identify regions bearing c-FOS expression. Next, we examined brain regions previously implicated in studies of anticipatory behaviors for food, drug or other rewards, where c-FOS was measured. Images were captured with a Nikon Eclipse E800 microscope (Nikon) equipped with a cooled CCD camera (Retiga Exi; Q-Imaging), using Q-capture software (RRID: SCR_014432, Q-Imaging) using the excitation wavelengths 480 ± 20 nm for Cy2, and 560 ± 40 nm for Cy3, with each channel acquired independently and then combined digitally. The localization of nuclei was determined using the mouse brain atlas of (Paxinos and Franklin, 2013). In addition, TH-ir was used to distinguish the nucleus accumbens shell from core and to delineate the ventral tegmental area (VTA) and substantia nigra. For large nuclei, an area in the mid-region of the nucleus was selected for analysis. Images were saved as .tiff files and imported into ImageJ Fiji (RRID:SCR_002285; Schindelin et al., 2012) to count c-FOS-ir cells. Two observers blind to the experimental conditions performed the cell counts. Inter-observer reliability was >90%.

The following regions were examined (listed from rostral to caudal, followed by the distance from bregma (Paxinos and Franklin, 2013) used to delineate that region); OB (3.17–2.77), prefrontal cortex (1.77–1.41), orbitofrontal cortex (OFC, 2.33–1.97), lateral septum (LS, 0.61–0.13), dorsal striatum (0.73–0.13), nucleus accumbens core and shell (1.21–0.73), ventral pallidum (0.61–0.25), bed nucleus of the stria terminalis (0.01 to 0.35), paraventricular nucleus of thalamus (-0.35 to 0.59), suprachiasmatic nucleus shell and core (−0.35 to −0.45), suprachiasmatic nucleus shell and core (−0.35 to −0.59), arcuate nucleus (−1.55 to −1.91), lateral hypothalamus (LH, −0.47 to −1.07), dorsomedial hypothalamic nucleus (DMH, −1.43 to −1.83).
to −1.91), ventromedial hypothalamic nucleus (−1.43 to −1.91), dentate gyrus of the hippocampus (DG, −1.79 to −2.45), insula (−0.59 to −1.23), habenula (−1.23 to −1.91), basolateral, basomedial, central, and medial amygdala (−1.07 to −2.03), ventral posteriomedial nucleus of the thalamus (−1.07 to −1.91), perifornix cortex (−1.07 to −1.91), periaqueductal gray −3.07 to −3.51), VTA, substantia nigra (−2.45), insula (−2.91 to −3.15), and supramammillary nucleus (−2.8 to −2.9). For each brain region, images taken from each side of three brain sections (six images) were scored for each animal, except for nuclei with a small rostro-caudal extent (paraventricular nucleus of thalamus, suprachiasmatic nucleus, supramammillary nucleus), where two brain sections (four images) were analyzed for each animal. c-FOS-positive cells in each brain region are reported as number of cells/mm².

Statistical analyses
Differences in anticipatory activity and in number of c-FOS-ir cells among the four groups (MA-ZT4, W-ZT4, MA-ZT10, and W-ZT10) were compared by two-way ANOVA (W or MA treatment) × (time of day), followed by Tukey post hoc test. Daily changes in anticipatory activity were evaluated by two-way repeated measures ANOVA. Correlation between activity levels and c-FOS was assessed by Pearson Product Moment. All analyses were done using SigmaStat 2.03 (RRID:SCR_010285, SPSS Inc.).

Results

Experiment 1: anticipatory behavior
The first goal was to assess the influence of treatment effects on anticipatory behavior and to determine whether the time of day modulated the anticipatory response to MA or water. During the experiments, nebulized MA or water was available in the nebulization chamber at either ZT4 or ZT10. The hypothesis was that an effect of time of day in the W group would point to a drug-independent circadian effect of anticipation, while a difference in responding at ZT4 versus ZT10 in the MA group would suggest a time of day effect of the drug. Interaction effects would suggest that the effect of MA is modulated by time of day, pointing to a role of circadian timing in anticipation of MA behavior. Figure 2 shows the daily activity of representative individuals over the entire experiment (Fig. 2A), these representative animals’ activity profiles during the last 10 experimental days (Fig. 2B), and for the group as a whole (Fig. 2C) summed over the last 10 experimental days. It is evident that after a few days of MA availability, mice develop anticipatory behavior, especially at ZT4 and much less at ZT10. Activity increased during and immediately after the time of MA nebulization, followed by a period of inactivity during the day. Nocturnal activity onset was not shifted by the skeleton photoperiod and during the nebulization period (Fig. 2D). Animals do not show anticipatory activity for available nebulized water, and the usual preference for activity during the nighttime is seen in all four groups.

Quantification of the results on locomotor activity before the availability of the material in the nebulization chamber, and total amount of daily activity is shown in Figure 3. The mice awaken and show more anticipation of MA availability before door opening at ZT4 than at ZT10, while they do not anticipate water availability at either time (Fig. 3A; main effects: MA vs water, F(1,22) = 23.52; p < 0.001; time of day, ZT4 vs ZT10, F(1,22) = 7.26; p = 0.014; interaction, F(1,22) = 7.23; p = 0.014). Although anticipatory activity for MA is greater at ZT4 than at ZT10, the amount of time spent in the nebulizing chamber does not differ between these groups (ZT4: 138.4 ± 25.5 s; ZT10: 128.0 ± 16.0 s; t(9) = 0.36, p = 0.73). Furthermore, there were no differences among groups in total amount of daily activity (Fig. 3B; main effect: MA vs water, F(1,22) = 0.68; p = 0.42; time of day, ZT4 vs ZT10, F(1,22) = 0.18; p = 0.68; treatment × time of day interaction, F(1,22) = 1.19; p = 0.29). This can be seen in the actograms of Figure 2, mice reduce nocturnal activity when they increase MA-associated diurnal activity, keeping total daily activity unchanged.

Experiment 2: c-FOS expression in brain
The behavioral data point to a main effect of treatment, time of day, and an interaction effect on anticipatory behavior. Thus, we aimed to identify brain areas that expressed c-FOS in the same manner, specifically those in which there was higher c-FOS expression with MA than water anticipation (drug effect), and more at ZT4 than ZT10 (time effect) and an interaction (anticipation effect). The c-FOS counts for all brain regions studied are shown in Figures 4B, 5B and Table 1. Statistical analysis (two-way ANOVA followed by the Tukey test) for the foregoing regional effects are shown in Table 2.

Two brain regions met all the aforementioned criteria: OFC (treatment: F(1,22) = 14.45 p = 0.001; time: F(1,22) = 11.56 p = 0.003; interaction: F(1,22) = 7.52 p = 0.013); DMH (treatment: F(1,22) = 9.99, p = 0.005; time: F(1,22) = 5.46, p = 0.031; interaction: F(1,22) = 5.15, p = 0.035). In the LS there were significant main effects, while interaction effects were marginally significant (treatment: F(1,22) = 30.81, p < 0.001; time: F(1,22) = 9.11, p = 0.007; interaction F(1,22) = 3.92, p = 0.063). In the OFC, c-FOS was densely expressed in the medial and ventral regions and more sparsely in the lateral region. c-FOS was expressed throughout the DMH. In the LS, c-FOS was expressed throughout the nucleus but more densely in the ventral region.

The results for these brain regions are shown in the photomicrographs (Fig. 4A) and the quantitative analysis is shown in the bar charts below the photomicrographs (Fig. 4B).

We also noted three brain regions, the OB, LH, and DG, where both the main effects of treatment and time of day were significant, but with no interaction effects (Fig. 5; Table 2). In the OB c-FOS was expressed in the glomerular, mitral and granule layers (OB: treatment: F(1,22) = 7.01, p = 0.016; time: F(1,22) = 17.29, p < 0.001; interaction: F(1,22) = 1.71, p = 0.207). In the LH, c-FOS expression occurred throughout the nucleus (treatment: F(1,22) = 12.3, p = 0.002; time: F(1,22) = 18.98, p < 0.001; interaction: F(1,22) = 5E−4, p = 0.982), and in the DG c-FOS was expressed in the granular layer (treatment: F(1,22) = 28.98,
Figure 4. A. Photomicrographs show c-FOS expression in brain areas where there is an effect of treatment, time of day and interaction in animals sacrificed at ZT4 or ZT10. Scale bars: 50 μm. B. Bar graphs show the number of c-FOS-positive cells in brain regions corresponding to the photomicrographs in A. Water = gray bars, MA = black bars; significant difference in Tukey tests where a, ZT4
c-FOS is not correlated with amount of locomotor activity

Finally, we examined whether the intensity of wheel-running itself could produce c-FOS expression by evaluating the correlation between amount of anticipatory locomotor activity (# wheel rotations before nebulization, data not normalized) and c-FOS expression (Table 3). In the water available groups, anticipatory activity ranged from 7.6 to 409.6 revolutions/h, and no significant correlations were found in any brain region. Similarly, in the MA groups, anticipatory activity ranged from 47 to 1800 revolutions/h, and there was no evidence of a correlation between activity and number of c-FOS-positive neurons, except for the piriform cortex, a brain region in which there was no effect of either time of day or treatment.

Discussion

Overview

A major function of the CNS is to anticipate and predict upcoming events. Our results present a number of novel findings on the nature of anticipation and its neural basis, evaluated in a noninvasive voluntary intake protocol. First, mice developed anticipatory behavior before the availability of MA but not in anticipation of nebulized water. When MA was available during the day, mice awoke during their normal sleep time and ran in the home cage wheel for ~2 h in anticipation of an upcoming interval of MA availability, with more anticipation in early versus late day. When nebulized water was available in the chamber, the mice did not develop anticipatory behavior. Three brain regions were implicated in the anticipatory response, namely the OFC, LS, and DMH. Here the patterns of c-FOS expression paralleled the anticipatory behavior. The present evidence points to CNS sites of cFos expression before MA availability, and highlights the usefulness of behavioral anticipatory responses in identifying activated brain regions. That said, the c-Fos expression seen here might reflect neural activity associated with or causal to behavioral anticipation, or causal to physiologic anticipation (e.g., autonomic outflow, peripheral hormones, etc.), or it might reflect neural activity in response to behavioral, endocrine, thermal or other anticipatory changes. These possibilities cannot be distinguished in the current experiment, nor are they necessarily separable.

The engagement of the OFC in MA anticipation is interesting as it has been implicated in the processing of signals involved in the reward value of odor, taste or touch (O’Doherty et al., 2000; Rolls, 2000). The OFC receives a dopaminergic projection from the VTA (Berger et al., 1991; Dunnett and Robbins, 1992). Variation in dopamine transporter function in OFC is associated with impulsive action (Yates et al., 2016). DA antagonists alter connectivity patterns in the OFC (Kahnt and Tobler, 2017) and decrease motivation for reward (Cetin et al., 2004). The OFC shows cFos activation in anticipation of a daily meal and, not surprisingly, even more activation when sucrose is added to the meal (Mitra et al., 2011). OFC has been implicated as a locus of relative value and of expected or outcomes (Izquierdo, 2017). Finally, the OFC has been implicated in drug addiction in preclinical and clinical studies (Schoenbaum and Shaham, 2008). Neural activation in the OFC increases in response to drugs (Volkow and Fowler, 2000; Guillem et al., 2017) and metabolic activity is proportional to the intensity of craving in humans (Volkow and Fowler, 2000). Taken together, the evidence suggests that the OFC can track the reward value of drugs and that its activity is associated with anticipation.

The finding that the DMH is involved in the timing of circadian responses is consistent with numerous reports in the food anticipation literature. (Angeles-Castellanos et al., 2004; Gooley et al., 2006; Poulin and Timofeeva, 2008; Mitra et al., 2011; Blum et al., 2012; Luna-Illades et al., 2017). The DMH has been implicated in feeding behavior and body weight regulation (Bellinger and Bernardis, 2002). However, the DMH does not seem to be the sole necessary brain nucleus for these responses, as DMH-lesions do not eliminate food anticipation (Landry et al., 2006; Landry et al., 2007). Activation of the DMH reflects the presence of food, evidenced by increases in c-FOS expression in anticipation of meals. This activation persists for several days at the previous time of food anticipation.
Figure 5. **A**, Photomicrographs show c-FOS expression in representative brain areas where there is an effect of both treatment and time of day but no interaction. Scale bars: 50 μm. **B**, Bar graphs show the number of c-FOS-positive cells in brain regions.
anticipation when ad libitum access to food is restored (Angeles-Castellanos et al., 2004; Blum et al., 2012). Orexin neurons in DMH may play a role in anticipation, as food anticipatory activity is significantly diminished in mice lacking orexin neurons (Akiyama et al., 2004). Furthermore, substantial evidence points to a role for orexin in modulating motivational, self-administration and reinstatement of drugs (James et al., 2017), possibly through actions on the mesolimbic dopamine system (Calipari and Espana, 2012).

Table 1. Number of c-FOS positive neurons in brain regions not shown in figures 4,5.

| Brain Regions | Regions with treatment effects | W-ZT4 | MA-ZT4 | W-ZT10 | MA-ZT10 |
|---------------|---------------------------------|-------|--------|--------|---------|
| Medial Amygdala | 27.4 ± 3.5                     | 51.4 ± 6.8 | 25.3 ± 3.9 | 38.1 ± 8.5 |
| Ventral Posterior-medial Thalamus | 13.9 ± 8.1 | 445.9 ± 141.9 | 9.5 ± 6.4 | 235.2 ± 123.8 |
| Regions with time of day effects | 316.1 ± 43.4 | 362.2 ± 52.2 | 246.4 ± 39.3 | 231.7 ± 44.4 |
| Paraventricular Nucleus of the Thalamus | 221.5 ± 27.0 | 190.4 ± 26.2 | 108.5 ± 23.5 | 114.2 ± 21.0 |
| Arcuate Nucleus | 19.8 ± 2.5                      | 16.7 ± 4.5 | 9.2 ± 3.6 | 10.5 ± 3.8 |
| Regions with no effects of treatment or time | 212.5 ± 27.9 | 142.8 ± 46.4 | 172.8 ± 67.6 | 205.4 ± 70.9 |
| Supra-optic Nucleus (Core) | 13.8 ± 4.0 | 19.4 ± 6.5 | 15.4 ± 4.6 | 15.1 ± 6.4 |
| Basolateral Amygdala | 20.9 ± 5.2 | 24.4 ± 6.7 | 15.8 ± 3.5 | 27.4 ± 9.0 |
| Central Amygdala | 27.7 ± 5.8 | 35.3 ± 4.1 | 24.5 ± 4.5 | 38.4 ± 12.3 |
| Peri-aqueductal gray | 27.5 ± 6.7 | 76.4 ± 40.1 | 38.4 ± 12.1 | 52.8 ± 22.5 |
| Piroform Cortex | 112.3 ± 37.4 | 214.6 ± 85.0 | 70.0 ± 21.3 | 145.8 ± 65.9 |
| Supra-mammillary nucleus | 38.4 ± 11.2 | 90.2 ± 30.5 | 59.6 ± 45.0 | 64.5 ± 25.6 |

Table 2. Statistical analysis of c-FOS expression in anticipation of access to nebulized MA- and water

| Brain area | Drug effect | Time of day effect | Drug x Time of day effect | Post hoc (Tukey test) |
|------------|-------------|--------------------|---------------------------|----------------------|
| Olfactory bulb | F₁,₁₂ = 7.01 p = 0.016 | F₁,₁₂ = 17.29 p < 0.001 | F₁,₁₂ = 1.71 p = 0.207 | p = 0.001 vs W; p = 0.013 vs MA; p = 0.344 vs MA-ZT4 |
| Orbitofrontal cortex | F₁,₁₂ = 14.45 p = 0.001 | F₁,₁₂ = 11.56 p < 0.001 | F₁,₁₂ = 7.52 p = 0.013 | p = 0.001 vs W; p = 0.005 vs MA; p = 0.452 vs MA-ZT4 |
| Lateral Septum | F₁,₁₂ = 30.81 p < 0.001 | F₁,₁₂ = 9.11 p < 0.001 | F₁,₁₂ = 3.92 p = 0.063 | p = 0.003 vs W; p = 0.001 vs MA; p = 0.018 vs MA-ZT4 |
| Lateral hypothalamus | F₁,₁₂ = 12.3 p = 0.002 | F₁,₁₂ = 18.98 p < 0.001 | F₁,₁₂ = 5.67 p = 0.025 | p = 0.008 vs W; p = 0.005 vs MA; p = 0.027 vs MA-ZT4 |
| Dorsomedial hypothalamic nucleus | F₁,₁₂ = 9.99 p = 0.005 | F₁,₁₂ = 5.46 p = 0.031 | F₁,₁₂ = 5.15 p = 0.035 | p = 0.005 vs W; p = 0.013 vs MA; p = 0.525 vs MA-ZT4 |
| Dentate gyrus | F₁,₁₂ = 28.98 p < 0.001 | F₁,₁₂ = 10.12 p < 0.001 | F₁,₁₂ = 1.98 p = 0.176 | p = 0.005 vs W; p = 0.001 vs MA; p = 0.010 vs MA-ZT4 |
| Medial amygdala | F₁,₁₂ = 9.43 p < 0.001 | F₁,₁₂ = 1.67 p = 0.212 | F₁,₁₂ = 0.88 p = 0.361 | p = 0.141 vs W; p = 0.012 vs MA; p = 0.139 vs MA-ZT4 |
| Ventroposteromedial thalamus | F₁,₁₂ = 13.57 p = 0.002 | F₁,₁₂ = 1.45 p < 0.001 | F₁,₁₂ = 2.24 p < 0.001 | p = 0.120 vs W; p = 0.004 vs MA; p = 0.083 vs MA-ZT4 |
| Paraventricular nucleus of the thalamus | F₁,₁₂ = 0.12 p = 0.73 | F₁,₁₂ = 5.03 p < 0.001 | F₁,₁₂ = 0.46 p < 0.001 | p = 0.058 vs W; p = 0.272 vs MA; p = 0.814 vs MA-ZT4 |
| Supra-optic nucleus | F₁,₁₂ = 0.28 p < 0.001 | F₁,₁₂ = 14.95 p < 0.001 | F₁,₁₂ = 0.58 p < 0.001 | p = 0.045 vs W; p = 0.383 vs MA; p = 0.868 vs MA-ZT4 |
| Shell | F₁,₁₂ = 0.11 | F₁,₁₂ = 0.79 | F₁,₁₂ = 0.45 | p = 0.083 vs W; p = 0.383 vs MA; p = 0.868 vs MA-ZT4 |
| Core | F₁,₁₂ = 0.07 | F₁,₁₂ = 0.06 | F₁,₁₂ = 0.37 | p = 0.549 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
| Arcuate Nucleus | F₁,₁₂ = 0.24 | F₁,₁₂ = 0.06 | F₁,₁₂ = 0.30 | p = 0.593 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
| Basolateral amygdala | F₁,₁₂ = 1.37 | F₁,₁₂ = 0.02 | F₁,₁₂ = 0.39 | p = 0.537 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
| Central amygdala | F₁,₁₂ = 1.94 | F₁,₁₂ = 0.06 | F₁,₁₂ = 0.17 | p = 0.684 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
| Periaqueductal gray | F₁,₁₂ = 2.60 | F₁,₁₂ = 0.06 | F₁,₁₂ = 0.11 | p = 0.813 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
| Piroform cortex | F₁,₁₂ = 0.85 | F₁,₁₂ = 0.01 | F₁,₁₂ = 0.94 | p = 0.455 vs W; p = 0.808 vs MA; p = 0.808 vs MA-ZT4 |
We found that c-FOS in the LS was activated more in MA than in W groups and more in early than late day, though interaction effects were marginally significant. A role for the LS in anticipation is consistent with work showing that MA seeking increases c-FOS expression in the LS (Cornish et al., 2012) and that withdrawal of self-administered MA is associated with activation of the septum, among other regions, suggesting a role in “craving” (Cornish et al., 2012). That LS stimulation is rewarding has long been known (Olds and Milner, 1954; Randt and Quartermain, 1972; Sheehan et al., 2004). The LS has connections with the mesocorticolimbic dopamine system, thereby regulating motivation. The LS can stimulate the activity of midbrain dopamine neurons and regulate the consequences of this activity on the ventral striatum (Sheehan et al., 2004). Prior work using c-FOS activation as a measure has strongly implicated the mesolimbic circuit in anticipatory behavior. The LS has also been implicated in addiction to several drugs of abuse: mice self-administer morphine into the LS, a response blocked by dopamine or opiate antagonists (Le Merrer et al., 2007). Inactivation of LS neurons attenuate context- and cue-induced reinstatement of cocaine seeking (McGlinchey and Aston-Jones, 2017) and inactivation of the Ca3-LS-VTA circuit blocks context-induced reinstatement of cocaine seeking (Luo et al., 2011; McGlinchey and Aston-Jones, 2017).

One puzzling aspect of the present work is that we have not found evidence of anticipation, using the c-FOS marker, in many of the brain areas identified in prior studies. A possible explanation can be species differences, as little of the prior work had been done on mice. Alternatively, there are substantial differences among studies in their experimental designs. Many prior studies examine food anticipation and it is generally accepted that food anticipation is affected by the size and the interval since the last meal (Strubbe and Woods, 2004), factors not involved in MA anticipation. However, even within studies of drugs, identification of brain regions mediating anticipation are mixed (Neisewander et al., 2000; Rhodes et al., 2005; Cornish et al., 2012; Li et al., 2015; McGlinchey and Aston-Jones, 2017). Differences between our results and the prior work on drug seeking also may be due to the fact that the latter animals had surgery for catheter placement, and performed bar-pressing tasks, or received daily injections, while our mice were free of handling and surgery and voluntarily left their home cage to seek MA.

### Novelty of our paradigm

As has been previously documented, experimenter-administered drug treatments are useful in uncovering mechanisms underlying MA effects in the brain. However, these procedures do not allow the exploration of reinforcing effects of MA, nor do they parallel human drug-taking behaviors (Krasnova et al., 2016). Intravenous MA self-administration protocols provide some face validity with respect to patterns of human MA intake, but retain the stresses associated with handling the animals and with the surgical procedures required for implants. A novel aspect of the present protocol is that the MA was available in a noninvasive voluntary intake protocol with animals living in their home cages. In these conditions, neural activation could be ascribed confidently to anticipatory responses, free from stresses associated with handling and surgery. Also, the MA was nebulized and inhaled by the mice; this is significant as intranasal administration represents the primary route of administration of MA for humans (Halkitis et al., 2003; Hart et al., 2008).

### Circadian effects

The use of timed daily MA administration permitted incorporation of circadian analytic tools in our study. It is well established that the pharmacological, physiologic and behavioral responses to drugs are impacted by the time of administration (Levy, 1991; Ballesta et al., 2017; Prosser and Glass, 2017). By examining two different times of day, the present work contributes to our understanding CNS mechanisms associated with the anticipation of a psychostimulant and its modulation by diurnal rhythms. Our results agree with previous work showing

### Table 3. Pearson product moment correlations between anticipatory wheel running and c-FOS expression

| Brain areas                                      | MA group (n = 11) | Water group (n = 12) |
|--------------------------------------------------|-------------------|----------------------|
| OB                                               | $r = 0.55 \ p = 0.08$ | $r = 0.28 \ p = 0.37$ |
| OFC                                              | $r = 0.55 \ p = 0.08$ | $r = 0.15 \ p = 0.64$ |
| LS                                               | $r = 0.25 \ p < 0.46$ | $r = 0.26 \ p = 0.42$ |
| LH                                               | $r = 0.37 \ p = 0.26$ | $r = 0.14 \ p = 0.66$ |
| DMH                                              | $r = 0.19 \ p = 0.58$ | $r = 0.29 \ p = 0.36$ |
| DG                                               | $r = 0.55 \ p = 0.08$ | $r = 0.01 \ p = 0.98$ |
| Medial amygdala                                  | $r = 0.06 \ p = 0.87$ | $r = 0.38 \ p = 0.22$ |
| Ventroposteromedial of thalamus                  | $r = 0.59 \ p = 0.06$ | $r = 0.08 \ p = 0.80$ |
| Paraventricular nucleus of thalamus              | $r = 0.49 \ p = 0.13$ | $r = 0.35 \ p = 0.27$ |
| Suprachiasmatic nucleus                          | $r = 0.15 \ p = 0.66$ | $r = 0.33 \ p = 0.30$ |
| Shell                                            | $r = 0.08 \ p = 0.82$ | $r = 0.11 \ p = 0.75$ |
| Core                                             | $r = 0.04 \ p = 0.89$ | $r = 0.08 \ p = 0.81$ |
| Basolateral amygdala                             | $r = 0.10 \ p = 0.91$ | $r = 0.12 \ p = 0.70$ |
| Basomedial amygdala                              | $r = 0.03 \ p = 0.94$ | $r = 0.33 \ p = 0.29$ |
| Central amygdala                                 | $r = 0.12 \ p = 0.73$ | $r = 0.47 \ p = 0.13$ |
| Periaqueductal gray                              | $r = 0.03 \ p = 0.44$ | $r = 0.47 \ p = 0.13$ |
| Piriform cortex                                  | $r = 0.13 \ p = 0.70$ | $r = 0.40 \ p = 0.20$ |

Cornish et al., 2012; Li et al., 2015; McGlinchey and Aston-Jones, 2017. Differences between our results and the prior work on drug seeking also may be due to the fact that the latter animals had surgery for catheter placement, and performed bar-pressing tasks, or received daily injections, while our mice were free of handling and surgery and voluntarily left their home cage to seek MA.
that circadian modulation of drug-seeking behavior and other rewards peaks in the early morning hours (Webb et al., 2009; Baltazar et al., 2013; Webb et al., 2015). The results are also consistent with a substantial body of work that point to circadian and diurnal rhythms throughout the brain (Harbour et al., 2013; Silver and Kriegsfeld, 2014; Frederick et al., 2017). Three brain regions, the OB, LH, and DG, showed increased c-FOS expression with significant differences between early and late day for both MA and W groups. We interpret these results to reveal the effects of MA on diurnal timing systems in these regions. Most neurons express circadian rhythms (Silver and Kriegsfeld, 2014) and thus it is not surprising to find that there are times of day effects in both MA and W groups. That said, highlighting endogenous diurnal responses, and how MA modulates them provides a tool for identifying drug effects.

Anticipatory activity to nebulized MA (but not W control) occurs not only in the voluntary access protocol, but is also seen when mice are placed daily by the experimenter into a chamber where MA was nebulized. Here too mice show more anticipatory activity in early versus late day (Keith et al., 2013). Similarly, they show more anticipatory activity in early day than in late day for free access to MA mixed with peanut butter (Keith et al., 2013). The preclinical evidence is consistent with clinical reports regarding drugs of abuse, in showing that admissions of overdose patients to the emergency department of urban hospitals are predominant in early evening, suggesting a diurnal effect (Raymond et al., 1992; Manfredini et al., 1994). In summary, behavioral studies consistently point to an important effect of time of day, in the absence of external cues, on the behaviors associated with many kinds of rewards, raising the question of the brain regions that are involved in anticipation.

**Endogenous circadian control**

Engagement of the endogenous circadian timing system is not under voluntary control, but the entrained responses of the brain and body develop over time, following repeated exposure (Fig. 2; Mistlberger, 1994). The evidence that circadian timing is involved is not simply that drugs administered at 24-h intervals produce circadian anticipatory activity that precedes daily drug availability by an hour or more, but also that anticipatory activity persists when the drug is withdrawn (Kosobud et al., 1998). The time of availability, at least in the case of food anticipation, is remembered for as long as two months following the initial exposure (Coleman et al., 1982; Yoshihara et al., 1997). The fact that anticipatory activity occurs even in SCN lesioned animals (Iijima et al., 2002) points to the possibility that circadian timing information may derive not only from extra-SCN brain sites but also from bodily signals (Honma and Honma, 2009; Patton and Mistlberger, 2013). Taken together with the previous work, the present results point to the important effect of the circadian timing system in behavioral and neural aspects of anticipation of MA. The results also suggest that MA can alter the amplitude of rhythmic neural responses.

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