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RESEARCH REPORT

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Segregation of caffeine reward and aversion in the rat nucleus accumbens shell versus core

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Abstract

Caffeine, the most commonly consumed psychoactive drug in the world, is readily available in dietary sources, including soft drinks, chocolate, tea and coffee. However, little is known about the neural substrates that underlie caffeine's rewarding and aversive properties and what ultimately leads us to seek or avoid caffeine consumption. Using male Wistar rats in a place conditioning procedure, we show that systemic caffeine at a low intraperitoneal dose of 2 mg/kg (or 100 μM injected directly into the rostral, but not caudal, portion of the ventral tegmental area) produced conditioned place preferences. By contrast, high doses of systemic caffeine at 10 and 30 mg/kg produced conditioned place aversions. These aversions were not recapitulated by a caffeine analog restricted to the periphery. Both caffeine reward and aversion were blocked by systemic D1-like receptor antagonism using SCH23390, while systemic D2-like receptor antagonism with eticlopride had smaller effects on caffeine motivation. Most important, we demonstrated that pharmacological blockade of dopamine receptors using α-flupenthixol injected into the nucleus accumbens shell, but not core, blocked caffeine-conditioned place preferences. Conversely, α-flupenthixol injected into the nucleus accumbens core, but not shell, blocked caffeine-conditioned place aversions. Thus, our findings reveal two dopamine-dependent and functionally dissociable mechanisms for processing caffeine motivation, which are segregated between nucleus accumbens subregions. These data provide novel evidence for the roles of the nucleus accumbens subregions in mediating approach and avoidance behaviours for caffeine.

KEYWORDS
dopamine, motivation, place conditioning, ventral tegmental area

Abbreviations: 8-SPT, 8-(p-sulphophenyl)theophylline; A1R, adenosine receptor subtype 1; A2R, adenosine receptor subtype 2; ANOVA, analysis of variance; cVTA, caudal ventral tegmental area; D1-like, dopamine receptor subtype 1; D2-like, dopamine receptor subtype 2; DA, dopamine; GABA, γ-aminobutyric acid; i.c., intracranial; i.p., intraperitoneal; KO, knockout; NAc, nucleus accumbens; NAcC, nucleus accumbens core; NAcSh, nucleus accumbens shell; PBS, phosphate-buffered saline; PFA, paraformaldehyde; rVTA, rostral ventral tegmental area; s.c., subcutaneous; TH, tyrosine hydroxylase; VTA, ventral tegmental area; α-flu, α-flupenthixol.

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1 | INTRODUCTION

Caffeine is a central nervous system stimulant that can increase arousal, improve mood and heighten attention (Brice & Smith, 2002; Lazurus et al., 2011; Lorist, Snel, Kok, & Mulder, 1996; Smith, 2009). Most of these effects, including those involving motivation, have been attributed to its role as a competitive adenosine receptor antagonist (subtypes A_1R and $A_{2A}R$) (Fredholm, Bättig, Holmén, & Zvartau, 1999; Higgins et al., 2007; Lazurus et al., 2011). However, despite the abundance of evidence implicating adenosine receptors in caffeine motivation (Bedingfield, King, & Holloway, 1998; El Yacoubi, Kedent, Parmentier, Costentin, & Vaugeois, 2005; Hilbert, May, & Griffin, 2013; Lazurus et al., 2011; Patkina & Zvartau, 1998), our group has demonstrated that the aversive effects of caffeine are spared in adenosine receptor mouse knockouts (KO) (Sturgess et al., 2010). Additionally, the rewarding effects of caffeine produced under conditions of dopamine (DA) receptor blockade in wild-type C57BL/6 mice were reproduced in A_1R KO, $A_{2A}R$ KO and double KO mice, indicating that adenosine receptor subtypes neither individually nor in tandem are necessary. This could be due to unknown compensatory mechanisms arising from transgenic manipulation. Nonetheless, downstream dopaminergic mechanisms were implicated in the aversive response to caffeine, as systemic pharmacological blockade of DA receptors reduced caffeine aversions (Sturgess et al., 2010). As caffeine may not act as an adenosine receptor antagonist to produce these motivational effects and does not bind directly to DA receptors as demonstrated by radioligand competitive binding assays (Watanabe & Uramoto, 1986), caffeine could be acting on an unknown receptor to produce a motivational response. However, since caffeine was found to mimic the activity of a DA receptor agonist within the brain (although presumably not acting directly on DA receptors), the ventral tegmental area (VTA) that contains a large population of DA neurons warrants further investigation (Nair-Roberts et al., 2008; Watanabe & Uramoto, 1986).

Evidence suggests that the VTA is important for rewarding behaviours, and many neuropsychiatric disorders, including substance abuse, are associated with disruptions to this brain region (Ashok, Mizuno, Volkow, & Howes, 2017; David, Segu, Buht, Ichaye, & Cazala, 2004; Laviolette & van der Kooy, 2003; Ting-A-Kee, Dockstader, Heinmiller, Grieder, & Kooy, 2009). However, growing evidence for its involvement in processing aversive stimuli raises the question of how the same brain region can elicit the opposing behaviours of approach and avoidance (Kim, Pollak, Hjelmstad, & Fields, 2004; Lammel et al., 2012; Pignatelli et al., 2017; Sellings, Baharnouri, McQuade, & Clarke, 2008). The downstream nucleus accumbens (NAc), composed of the anatomically and functionally dissociable medial shell and lateral core subregions, provide one conceivable way by which information encoding approach and avoidance behaviour can be separated. Although both NAc subregions are reciprocally connected to the VTA, the shell strongly projects to the ventromedial part of the subcommissural ventral pallidum and the pre-optic area/lateral hypothalamus, while the core projects to the dorsolateral ventral pallidum and the medial substantia nigra (Heimer, Zahm, Churchill, Kalivas, & Wohltmann, 1991; Zahm, 1999; Zahm & Heimer, 1990, 1993). These two subregions also differ functionally in mediating the approach and avoidance behaviours elicited by drugs such as nicotine and amphetamine (Ito & Hayen, 2011; Sellings et al., 2008). For example, 6-hydroxydopamine lesions of the medial NAc shell (referred to as the "shell" in the present study) reduced nicotine-conditioned place preferences, while lesions of the NAc core abolished nicotine-conditioned taste aversions (Sellings et al., 2008).

In view of the opposing roles of the NAc shell and core in modulating the motivational valence of drugs of abuse, we used a place conditioning procedure to examine the potential role of these subregions in processing the rewarding (indicated by a relative increase in conditioned place preference) and aversive (indicated by a relative increase in conditioned place aversion) effects of acute caffeine. We sought to determine whether there are separate, independent neural pathways between motivationally driven behaviours (caffeine reward versus aversion) and neural substrates (NAc shell versus core).

2 | MATERIALS AND METHODS

2.1 | Subjects

All animals were handled in accordance with the regulations and guidelines of the University of Toronto Animal Care Committee, the Animals for Research Act in Ontario and the Canadian Council on Animal Care. Adult male Wistar rats (weighing 250–350 g) were purchased from Charles River (Montreal, Canada) and maintained in the animal facilities of the University of Toronto Division of Comparative Medicine. Each rat was used in only one experimental condition with no overlap of data sets, and the total number of rats used in all studies was 293. As systemic caffeine self-administration in rodents is inconsistent (Griffiths & Woodson, 1988) and it is known that there are sex differences related to both caffeine consumption (Noschang et al., 2009) and place conditioning (Russo et al., 2003), female rats were not included in these studies. The rats were pair-housed with environmental enrichments (plastic tunnel, wooden block and chew toy) in clear Plexiglas cages at a constant temperature of 22°C on a controlled 12-hr light/dark cycle (lights on at 7 a.m.). Rats were given ad libitum access to food and water. Rats did not receive any drugs prior to the first day of conditioning.

2.2 | Drugs

All drugs used were dissolved in saline with the final pH of the solution adjusted to 7.4. Caffeine (C0750, Sigma-Aldrich) was dissolved in warm saline and a dose–response curve for the motivational effects of intraperitoneal (i.p.) injections of caffeine was generated in the present study with doses ranging from 1 mg/kg to 60 mg/kg. Bilateral intracranial injections (i.c.) of caffeine (10, 100 and 1,000 μM) were performed through surgically implanted cannulas (Plastics One) targeting the VTA (0.5 μl per hemisphere, administered over 1 min). The injector tip was kept in place for an additional minute to ensure complete diffusion of the drug from the tip of the injector. The caffeine analog, 8-(p-sulphophenyl)theophylline (8-SPT, sc-217511; Santa Cruz), was administered via i.p injections at a dose of 10 mg/kg only. The non-selective DA receptor antagonist, α-flupenthixol (α-flu, F114; Sigma-Aldrich), was administered as a pretreatment via i.p. injections (0.8 mg/kg, 2.5 hr prior to conditioning) or bilateral i.c. injections (3 μg/0.5 μl per hemisphere in the NAc, 15 min prior to conditioning). The doses and timings of injections were selected based on previous studies showing that α-flu could antagonize postsynaptic DA (both D1-like and D2-like subtypes) receptors (Creese, Burt, & Snyder, 1976), without producing any motivational effects of its own (Laviolette & van der Kooy, 2003). The D1-like receptor antagonist, SCH23390 (D054; Sigma-Aldrich), was injected at a dose of 0.05 mg/kg subcutaneously (s.c.), 10 min prior to conditioning. At this dose and injection timing, SCH23390 is not intrinsically motivating, but can block the rewarding effects of morphine, nicotine, amphetamine and diazepam, and the aversive effects of naloxone, phencyclidine and picrotoxin in place conditioning paradigms (Acquas, Carboni, Leone, & Chiara, 1989; Leone & Di Chiara, 1987). The D2-like receptor antagonist, eticlopride (E101; Sigma-Aldrich), was injected at a dose of 0.1 mg/kg i.p., 15 min prior to conditioning, which is sufficient to block preferences for amphetamine and novel objects (Bevins et al., 2002; Hoffman, 1994).

2.3 | Surgical cannulation

To target specific brain regions, rats were surgically cannulated under inhaled anaesthesia (2%–5% isoflurane). Rats were anaesthetized in a small chamber, and the fur between the ears of the rat was shaved to expose the scalp. The heads of the rats were fixed in place by the stereotaxic frame (with nose cone connected to the anaesthetic machine) on top of a heat pad to maintain body temperature. The rats were injected with analgesic (ketoprofen, 5 mg/kg s.c.). The skin of the shaved area and the surrounding fur were cleaned with two sets of alternating ethanol and iodine treatment. The shaved skin was cut away to reveal Bregma and Lambda on the skull. Small burr holes were drilled into the skull based on coordinates (in mm from Bregma) for the rostral VTA (AP: rostral to −5.5, ML: ± 0.5, and DV: −7.6), caudal VTA (AP: caudal to −5.5, ML: ± 0.5, and DV: −7.6), medial NAc shell (AP: +1.3, ML: ±1.0, and DV:-7.4) or lateral NAc core (AP: +1.3, ML: ± 2.4, and DV: −7.4) (Olson & Nestler, 2007; Olson et al., 2005; Paxinos & Watson, 2005). The injections for Figure 3b were targeted for the border of the NAc shell and core, and postmortem histology confirmed no clear anatomical distinction for separation into shell and core groups. There was not enough variation in the injections in the VTA (Figure 3c) and not enough animals in the group to divide into rostrocaudal groups. Four jeweller's screws (0–80 X 3/32; Plastics One) were screwed into the skull in positions neighbouring the burr holes. One stainless steel guide cannula (C313G(2)-G11/SP; Plastics One) that branched into two cannulas (to target the two brain hemispheres) was inserted through the burr holes to target either the NAc or VTA. Two guide cannulas were inserted if the target was both the NAc and VTA. The tips of the guide cannulas were positioned 1 mm above the target brain region. Dental acrylic paste was carefully applied to cover the exposed skull area, the jeweller's screws, and midway (to prevent occlusion) of the exposed portion of the guide cannulas. After the dental acrylic hardened, removable dummy cannulas (C313DC/1/SPC; Plastics One) were inserted into the guide cannulas to prevent occlusion over the course of surgical recovery and behavioural experiments. For post-operative care, antibiotic ointment (Polysporin) was applied to the exposed surgical area. Rats were put into a clean cage with mash and given 1 to 2 hr to recover from the surgery under a heat lamp. Rats were checked daily by the animal facility staff for surgical wound cleaning, proper hydration, signs of infection and overall health. Rats were given at least one full week (up to three weeks) to recover from the surgeries before further experimentation. During conditioning, the dummy cannulas were removed from the guide cannulas and removable internal cannulas (C313I/SPC; Plastics One) attached to an infusion apparatus were inserted into the guide cannulas with the tips of the internal cannulas extending 1 mm ventral to the guide cannula tips. Shortly after each infusion, the internal cannulas were removed and the dummy cannulas re-inserted.

2.4 | Place conditioning procedure

Prior to place conditioning, the rats were habituated for 20 min in a neutral, grey chamber (41 x 41 x 38 cm box) to reduce novelty effects. The rats then underwent a place conditioning procedure, which was fully counterbalanced in terms of drug-place pairing and day of first drug exposure. Conditioning alternated between two place conditioning

boxes (41 x 41 x 38 cm boxes) that were distinct in colour, floor texture and scent. One box had black walls, had a smooth black Plexiglas floor and was scented with ~0.3 ml of diluted glacial acetic acid (AX0073; EMD) solution. The solution was 12% by volume acetic acid, diluted with water. The other box had white walls, had a metal grid wire floor over a smooth metallic surface and was unscented. The amount of acetic acid was determined by testing a separate group of rats from the same cohort to ensure that there was no initial baseline preference (Mucha, Kooy, O'Shaughnessy, & Bucenieks, 1982). These rats were conditioned to vehicle (saline) only in both box types over 8 days for 40 min each day. During the conditioning period, rats were separated into vehicle-pretreated (saline) or drug-pretreated groups (DA antagonism with α-flu, SCH23390, or eticlopride), and administered i.c., s.c., or i.p. where appropriate. Rats in experimental conditions requiring pretreatment received pretreatment on all conditioning days, regardless of drug or vehicle conditioning session. After pretreatment (if applicable), treatment drug (caffeine or 8-SPT) and vehicle (saline) solutions were administered and rats were immediately exposed to the conditioning boxes for a total of 40 min each session. Forty minutes is a sufficient period to capture peak caffeine levels in the brain following i.p. injections (Latini, Bonati, Castelli, & Garattini, 1978; Lukas, Brindle, & Greengard, 1971) and 8-SPT has a half-life of about 14 min in rats (Wormald, Bowmer, Yates, & Collis, 1989). Between 48 to 96 hr following the final conditioning session, rats were tested in a rectangular testing box consisting of two compartments with features identical to the conditioning boxes separated by a neutral, grey zone. On testing day, rats were placed on the centre grey zone and allowed to roam freely in the testing box for 10 min under a mounted camera. All rats were tested in a drug-free state. The amount of time spent on each side of the testing box was recorded by an experimenter viewing the recordings and therefore, blinded to drug conditions. A greater relative amount of time spent in the previously drug-paired compartment indicated a

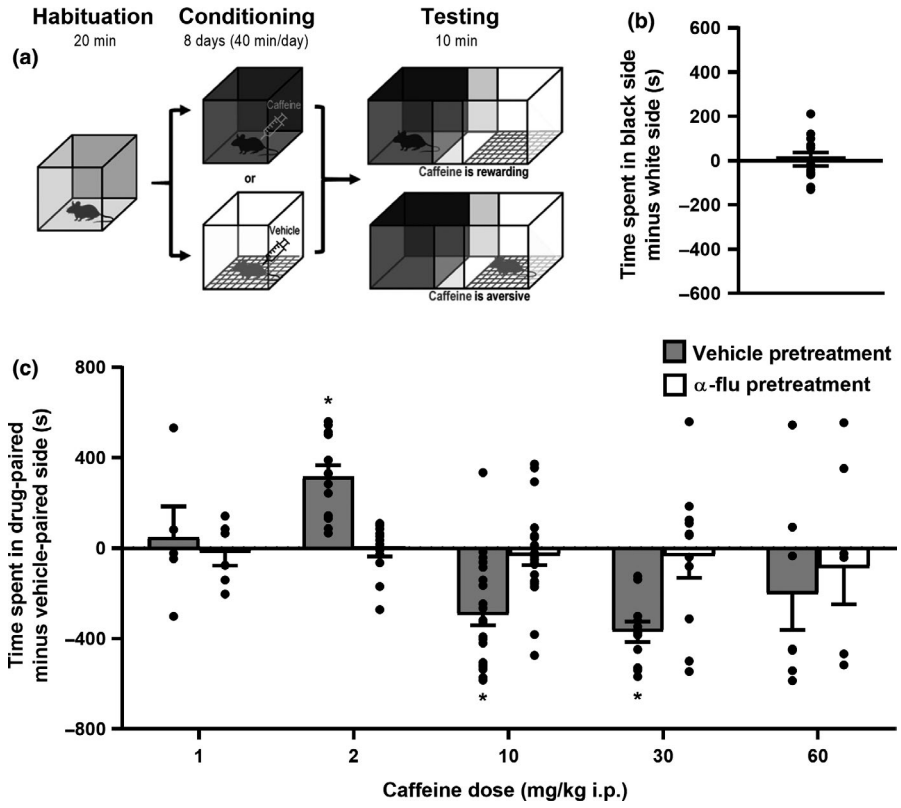

FIGURE 1 Systemic caffeine produces DA-dependent rewarding effects at low doses, and DA-dependent aversive effects at high doses. (a) Schematic of the place conditioning paradigm (counterbalanced for which environment is paired with caffeine, and for whether caffeine or saline is given first) and its timeline. (b) Baseline preferences for each side of the testing apparatus were found to be similar in a separately tested group of animals, that were given only saline during the conditioning phase. (c) In the systemic caffeine dose–response curve, there was a significant dose by pretreatment interaction, confirming that α-flu pretreatment (white bars) disrupted the motivational effects of caffeine. At a dose of 2 mg/kg i.p of caffeine, the vehicle-pretreated animals (grey bars) experienced a significant caffeine reward, which was blocked by α-flu pretreatment. At 10 and 30 mg/kg i.p., animals experienced significant aversions, which could be blocked by α-flu pretreatment. Bars represent the mean amount of time that animals spent in the two sides (black minus white side, and drug-paired minus vehicle-paired side) of the testing apparatus ± *SEM*s. *$p < .05$ for comparisons of mean difference scores compared to zero

conditioned place preference, while a greater relative amount of time spent in the previously vehicle-paired side indicated a conditioned place aversion.

2.5 | Verification of cannula placements

Following testing, only the rats that had received i.c. cannulations were deeply anaesthetized with 54 mg/kg i.p. of sodium pentobarbital (Ceva Santé Animale). Rats were perfused with ~100 ml of phosphate-buffered saline (PBS), followed by ~100 ml of 4% paraformaldehyde (PFA, P6148, Sigma-Aldrich) dissolved in PBS. Brains were removed and preserved in 4% PFA solution overnight, and then transferred to a 30% sucrose (BioShop) solution stored at 4°C. Coronal sections of flash-frozen brains were cut at a thickness of 40 μM with a freezing microtome (Cryostat model Hm525 NX; Thermo Scientific). The sectioned slices were mounted on slides and stained with Cresyl violet (C5042; Sigma-Aldrich). The slides were viewed under a light microscope to verify the bilateral cannula placements in the NAc and VTA.

2.6 | Statistical analysis

All analyses were conducted using GraphPad Prism software, with no overlap of data sets. All results (except for Figures 1b and 2b,d) are displayed as the mean time spent in drug-paired minus vehicle-paired side ± *SEM*s. Differences were considered significant if the *p*-value <.05. Every analysis of variance (ANOVA) conducted was followed by *post hoc* Tukey's multiple comparison tests. A two-way ANOVA was conducted for the systemic caffeine dose–response

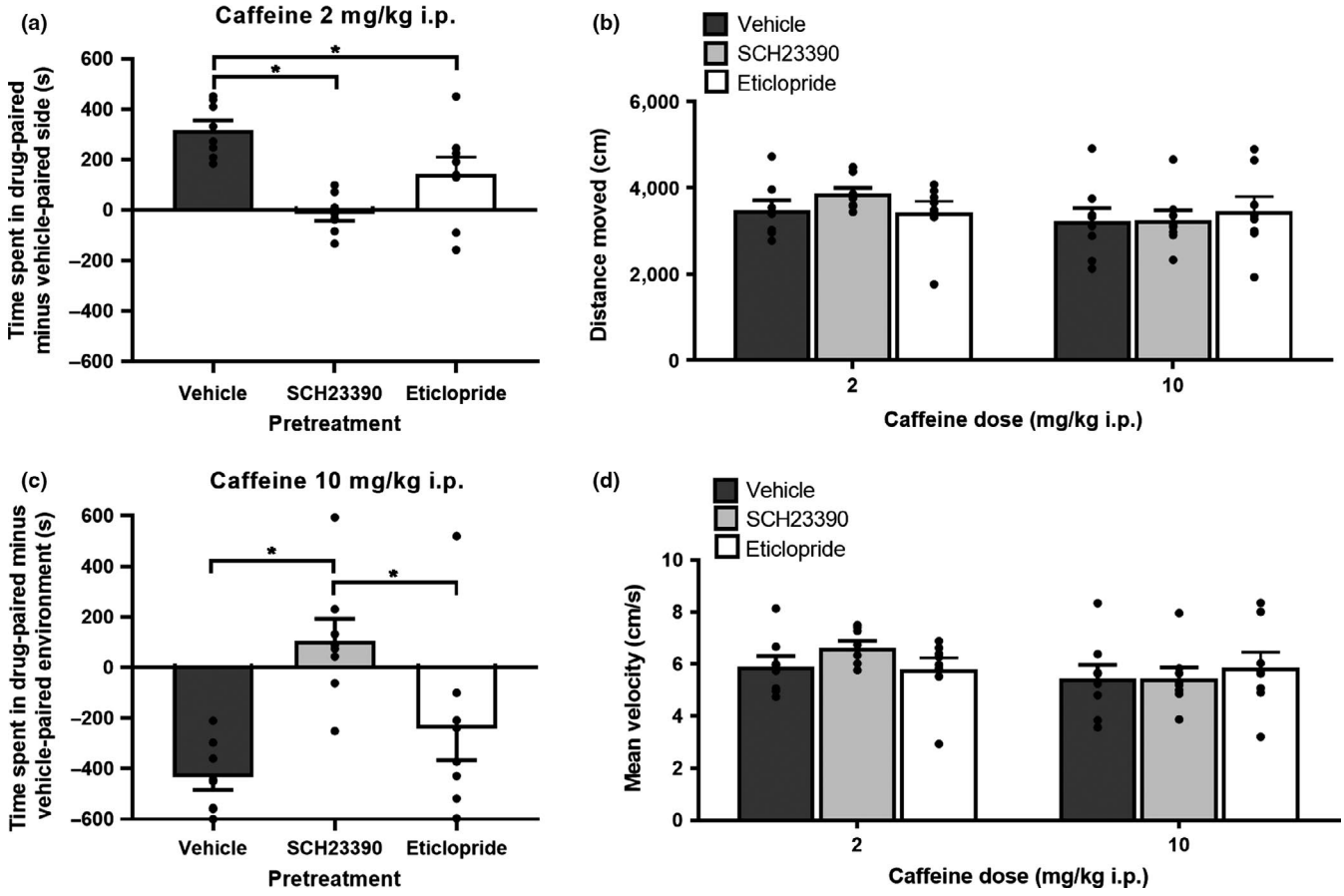

FIGURE 2 Systemic caffeine reward and aversion are both dependent on D1-like receptor activity. (a) In animals given 2 mg/kg i.p. caffeine, systemic injections of SCH23390 blocked place preferences for caffeine, while systemic injections of eticlopride reduced preferences. (b) Locomotor activity, indicated by the total distance travelled by the animals, was not different significantly between the animals given 2 or 10 mg/kg i.p. caffeine. (c) In animals given 10 mg/kg i.p. caffeine, systemic injections of SCH23390 blocked place preferences for caffeine. (d) Locomotor activity, indicated by the mean velocity of animals, was not different significantly between the animals given 2 or 10 mg/kg i.p. caffeine. Bars represent the mean amount of time that animals spent in the drug-paired minus vehicle-paired sides of the testing apparatus, the mean total distances moved, or the mean velocities ± *SEM*s *$p < .05$

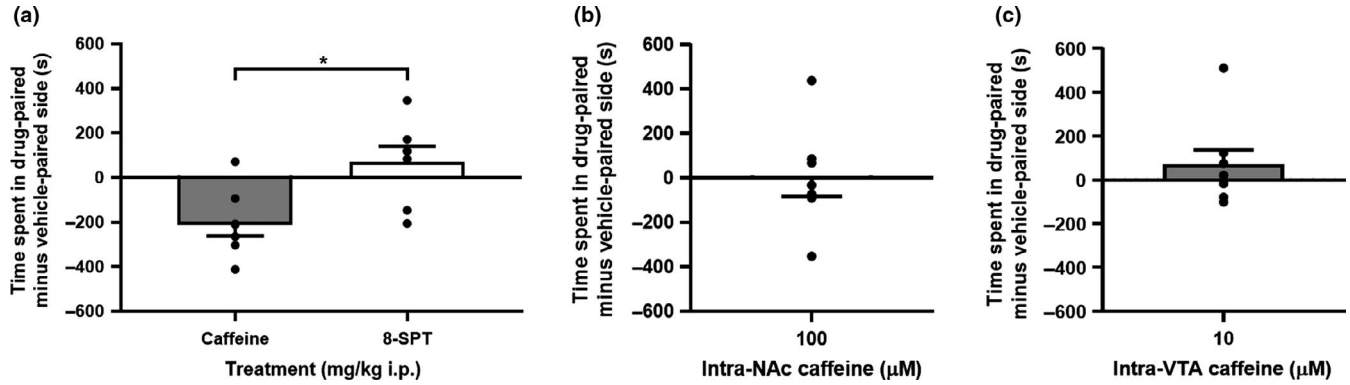

FIGURE 3 Systemic caffeine aversion is centrally mediated, but caffeine in the NAc or VTA (at a low dose) alone is insufficient to produce aversion. (a) Animals treated with caffeine (10 mg/kg i.p.) showed aversions that were not recapitulated by the administration of 8-SPT (10 mg/kg i.p.). (b) Animals given caffeine (100 μM) directly in the NAc did not exhibit any behaviours indicating preference or aversion. (c) Animals given caffeine at concentrations of 10 μM directly in the VTA did not exhibit any behaviours indicating preference or aversion. Bars represent the mean amount of time that animals spent in the drug-paired minus vehicle-paired sides of the testing apparatus ± *SEM*s. *$p < .05$

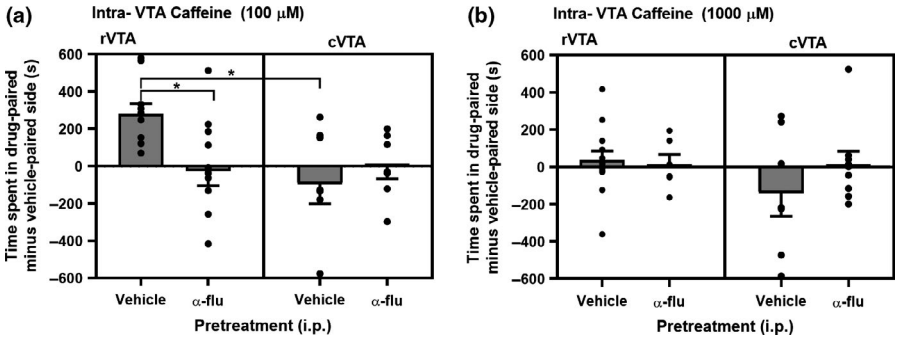

FIGURE 4 Caffeine reward is mediated through the rostral, not caudal VTA. (a) Intra-VTA (100 μM) caffeine injections produced conditioned place preferences when specifically targeted to the rVTA. In the cVTA groups, animals spent similar amounts of time in both sides of the testing apparatus without or with α-flu pretreatment. (b) At 1,000 μM of caffeine in the VTA, animals spent similar amounts of time in each side of the testing apparatus in the rVTA and cVTA groups with or without α-flu i.p. pretreatment. Bars represent the mean amount of time that animals spent in the drug-paired minus vehicle-paired sides of the testing apparatus ± *SEM*s. *$p < .05$

curve (Figure 1c). For Figure 1, comparisons of the mean difference scores to zero were conducted with one-sample *t* tests, with *p*-values adjusted using the Bonferroni correction. One-way ANOVAs were conducted for the experiments testing the effects of SCH23390 and eticlopride on conditioned place preferences (Figure 2a,c). A two-way ANOVA was used to compare mean distances travelled and mean velocity across caffeine doses (Figure 2b,d). A Student's *t* test was conducted for the experiment comparing the effects of 8-SPT and caffeine (Figure 3a). For Figure 3b,c a one-sample *t* tests were performed to compare the mean difference scores to zero. Two-way ANOVAs were conducted for the experiments comparing intra-VTA injections specifically to the rVTA and cVTA with 100 μM and 1,000 μM of caffeine (Figure 4). A one-way ANOVA was conducted for the experiments comparing the effects of vehicle and α-flu pretreatment in the NAc core and shell. Animals with mistargeted cannula placements (e.g. mistargeted in one hemisphere and complete misses, $n = 8$ in total) were excluded from statistical analyses.

3 | RESULTS

3.1 | Systemic caffeine elicits DA-dependent reward at low doses and aversion at higher doses

To examine the behavioural response to systemic caffeine in male rats, a place conditioning paradigm was used (Figure 1a). First, a group of adult male rats were tested for baseline bias towards either conditioning box type to ensure that changes in preference were due to drug-place conditioning. After confirming that the boxes were balanced and that the rats had no preference for either box type (Figure 1b, one-sample *t* test, $p = .359$, $n = 12$), we randomly assigned new cohorts of rats to conditioning groups for unbiased place conditioning (Mucha et al., 1982; Simpson & Riley, 2005). A dose–response curve for systemic caffeine using doses ranging from 1 mg/kg to 60 mg/kg i.p was generated (Figure 1c). A two-way ANOVA revealed a significant dose by pretreatment interaction,

confirming that α-flu pretreatment disrupted the motivational effects of caffeine ($F_{4,109} = 7.990$, $p = .0001$). At a dose of 2 mg/kg i.p of caffeine, a comparison test of the mean difference score to zero indicated that the non-pretreated animals experienced a significant caffeine reward ($p = .001$, $n = 13$). At 10 mg/kg i.p. ($p = .001$, $n = 24$) and 30 mg/kg i.p. ($p = .001$, $n = 11$), caffeine produced significant aversions, which were not significantly different from each other ($p = .9999$). The difference scores between the time that the animals spent in the drug-paired compartment minus the vehicle-paired compartment at caffeine doses of 10 and 30 mg/kg i.p. were not significantly different (Figure 1c). Therefore, the dose of 10 mg/kg i.p. was used for the remainder of this study when examining the aversive effects of caffeine.

We hypothesized that caffeine aversions would diminish in response to DA receptor antagonism based on our previous work showing this to be the case in mice (Sturgess et al., 2010). Comparable to the results seen in mice, pretreatment with a DA receptor antagonist (α-flu, 0.8 mg/kg i.p.) blocked the conditioned place aversions produced by 10 and 30 mg/kg i.p of caffeine in rats (10 mg/kg: $p = .001$, $n = 24$; 30 mg/kg: $p = .001$, $n = 11$).The pretreatment with α-flu also blocked the conditioned place preferences when a low dose of 2 mg/kg i.p. of caffeine was administered ($p = .9999$, $n = 13$). Thus, the acute caffeine reward and aversion in rats as elicited by systemic administrations of low and high doses, respectively, are dependent on dopaminergic transmission.

3.2 | Systemic caffeine reward and aversion are primarily dependent on D1-like receptor activity

A selective D1-like receptor antagonist (SCH23360) and a selective D2-like antagonist (eticlopride) were used to investigate which DA receptor family was responsible for systemic caffeine reward and aversion. In animals given 2 mg/kg i.p. caffeine, a one-way ANOVA ($p = .0003$, $F_{2,21} = 12.34$) revealed that systemic injections of SCH23390 blocked place preferences for caffeine ($p = .0002$, $n = 8$) while systemic injections of eticlopride reduced preferences ($p = .0475$, $n = 8$). In animals given 10 mg/kg i.p. caffeine, a one-way ANOVA ($p = .0015$, $F_{2,21} = 9.003$) revealed that systemic injections of SCH23390 blocked place preferences for caffeine ($p = .0013$, $n = 8$) while systemic injections of eticlopride had no significant effect ($p = .4608$, $n = 8$). As both caffeine reward and aversion were blocked completely by antagonizing D1-like receptors (Figure 2a,b) and D2-like receptor antagonism mildly reduced caffeine place preferences (Figure 2a), α-flu was used for the following experiments for its relative non-selectivity for dopamine receptors.

Both caffeine and DA antagonists are known to affect locomotion (Garrett & Holtzman, 1994), and residual effects from injections that occurred during the conditioning phase could potentially confound the measurement of place preferences during the testing phase. For example, rats may spend more time in one chamber due to the reduced ability to move rather than a preference for that chamber. Locomotor activity, measured by the total distances travelled by the rats and their mean velocities, was tracked during the testing phase to ensure that no lingering locomotive effects were present (Figure 2b,d). These measures were not significantly different between the animals given 2 or 10 mg/kg i.p. caffeine (distance: two-way ANOVA, $F_{1,42} = 1.892$, $p = .1762$; velocity: two-way ANOVA, $F_{1,42} = 2.084$, $p = .1563$). As neither the locomotor-stimulant effects of caffeine nor the locomotor-depressant effects of DA antagonists were observed at testing, these factors are unlikely to confound or influence the expression of place preference or aversion (Figure 2b,d).

3.3 | Systemic caffeine aversion is centrally mediated, but caffeine directly in the NAc and VTA is insufficient to produce aversion

Next, we asked whether the aversive effects of high doses of systemic caffeine were solely mediated by the central nervous system or if caffeine-responsive adenosine receptors in the peripheral nervous system also contributed. To test this, a non-selective adenosine receptor antagonist, 8-SPT, that has similar effects to caffeine (Evoniuk, Borstel, & Wurtman, 1987), was administered peripherally at 10 mg/kg i.p. in an identical place conditioning paradigm as the systemic caffeine experiments described above. When administered peripherally, 8-SPT remains undetectable in the brain at doses as high as 50 mg/kg i.p (Evoniuk et al., 1987). Despite 8-SPT having greater binding affinity to adenosine receptors than caffeine (Sturgess et al., 2010; Tao & Abdel-Rahman, 1993), there was no significant difference in the time that the rats spent in the vehicle- or 8-SPT-paired sides at testing (Figure 3a). When compared to with caffeine at 10 mg/kg i.p., there is a significant difference (t test, $p = .0065$, $n = 15$). This suggests that peripheral activation of adenosine receptors alone at 10 mg/kg i.p. does not contribute to the behavioural expression of caffeine aversions.

To determine where caffeine motivation may be mediated within the brain, the mesolimbic pathway from the VTA to NAc was chosen for its well-established role as a drug motivation circuit (Laviolette & van der Kooy, 2003; Nader & van der Kooy, 1997; Nestler, 2005). Caffeine was injected directly into the rat NAc immediately preceding place conditioning, but did not affect conditioned place preference behaviour significantly (one-sample t test, $p = .9574$, $n = 8$) (Figure 3b).

Caffeine (10 μM) injected non-specifically in the VTA also did not affect place preference behaviour (one-sample *t* test, $p = .3463$, $n = 8$) (Figure 3c). This suggested either caffeine in the VTA had no effect on motivation or the amount of caffeine was not sufficient. To follow-up with the latter possibility, caffeine was administered at a concentration of 100 μM and 1,000 μM in the rostral and caudal VTA.

3.4 | Caffeine injections into the rostral VTA elicit reward

Rostral VTA (rVTA) and caudal VTA (cVTA) were defined based on Olson et al.' (2005) study (rVTA defined rostral to −5.55 mm Bregma and cVTA defined as caudal to −5.55 mm Bregma). This rVTA area is defined to include the anatomical VTA subregions composed of the rostral subregion and the rostral portion of the parabrachial pigmentosus (Paxinos & Watson, 2005). Indeed, a significant interaction (two-way ANOVA, $F_{1,34} = 6.031$, $p = .0193$) of pretreatment (vehicle i.p. versus. α-flu i.p.) by VTA subregion (rVTA versus. cVTA) was revealed when caffeine was injected at a concentration of 100 μM (Figure 4a). Given that the VTA is composed of a majority of dopaminergic neurons (Nair-Roberts et al., 2008) and that systemic caffeine at 2 mg/kg i.p. was able to produce DA-dependent (both D1-like and D2-like-mediated) reward, we injected rats with α-flu (0.8 mg/kg i.p) to determine whether DA activity was also required for intra-rVTA caffeine reward. Multiple comparison tests indicated that caffeine (100 μM) directly injected into the rVTA only could elicit a significant reward that could be disrupted by α-flu pretreatment ($p = .0320$, $n = 10$, $n = 9$) (Figure 4a). In the cVTA groups, animals spent similar amounts of time in both sides of the testing apparatus without or with α-flu pretreatment ($p = .8695$, $n = 9$, $n = 7$) (Figure 4a). This suggests that rVTA caffeine at a 100 μM concentration can reproduce the place preference behaviour of rats given 2 mg/kg i.p. of caffeine (Figure 1c). It seems unlikely that this blockade is due to non-specific effects of α-flu, such as those on learning and memory. This same dose of α-flu does not block the rewarding effects of opiates or food in previously drug naïve or food-sated animals, respectively (Bechara & van der Kooy, 1992; Laviolette, Nader, & Kooy, 2002). Additionally, α-flu does not block the aversive effects of lithium chloride (Laviolette & van der Kooy, 2003).

When caffeine was injected at a higher concentration of 1,000 μM in the VTA, there was no interaction between pretreatment and VTA subregion (two-way ANOVA, $F_{1,31} = 1.252$, $p = .2717$) (Figure 4b). Injections of caffeine (1,000 μM) into the cVTA hinted at aversion (Figure 4b), similar to the results seen in the caffeine i.p. dose–response curve where aversions could be produced with 10 and 30 mg/kg i.p. doses of caffeine (Figure 1c). This is consistent with the growing evidence for the role of the cVTA in mediating aversive motivational responses (Balcita-Pedicino, Omelchenko, Bell, & Sesack, 2011; Bourdy & Barrot, 2012; Sanchez-Catalan et al., 2017).

3.5 | Caffeine injections into the rVTA elicit reward that is mediated by the NAc shell, but not core

Having determined that rVTA DA neurotransmission is necessary for caffeine reward, the roles of the downstream NAc subregions were investigated. While increased DA levels in the NAc shell are associated with increased caffeine locomotion and arousal (Lazarus et al., 2011; Solinas et al., 2002), the role of both NAc subregions in the DA-mediated motivational effects of caffeine is unknown. Cannulas were implanted in the rVTA and NAc, targeting either the shell or core (placements shown in Figure 5). To determine which NAc subregion is necessary for the

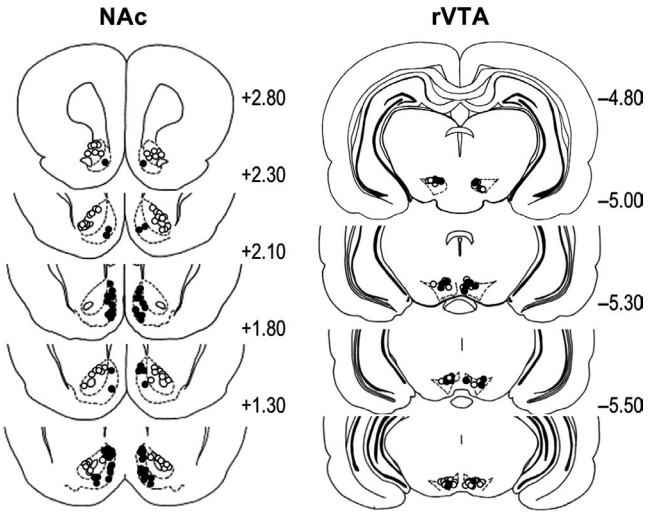

FIGURE 5 Verification of bilateral cannula placements. Bilateral cannula tips targeted at the medial NAc shell (●), lateral NAc core (○) and rostral VTA for data shown in Figure 6. The open and closed circles in the rVTA refer to their NAc shell and core counterparts. If the experimental group targeted both the rVTA and NAc shell, then both were marked with a closed circle. If the experimental group targeted both the rVTA and NAc core, then both were marked with a closed circle. The outlines shown in the rVTA slices represent the anatomical VTA subregions, composed of the rostral VTA (VTAR) and the rostral portions of the parabrachial pigmentosus. For Figure 6a,b, caffeine was injected in the rVTA following pretreatment of intra-NAc shell or intra-NAc core injections of α-flu or vehicle. For Figure 6c,d, caffeine was administered i.p. following α-flu or vehicle pretreatment in the NAc shell or NAc core. Shown in figure are coronal slice reconstructions based on the stereotaxic atlas of Paxinos and Watson (2005) with reference to Bregma. Numbers indicate the approximate distance from Bregma in millimetres.

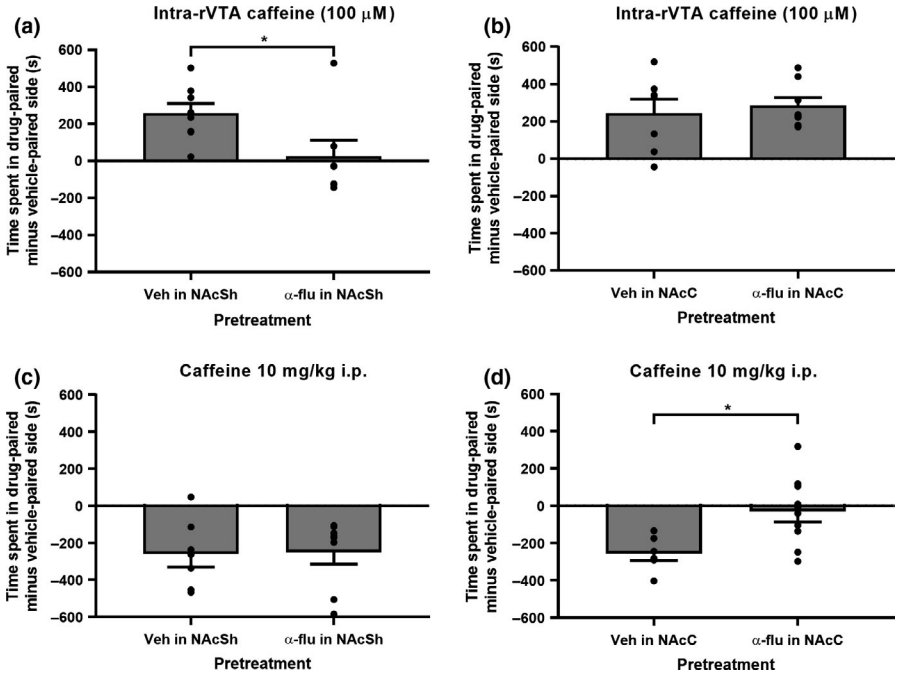

FIGURE 6 Intra-rVTA caffeine reward and systemic (10 mg/kg i.p.) caffeine aversion are both DA-dependent, but segregated in the NAc shell and NAc core, respectively. (a) Intra-NAc shell (NAcSh in graph) injections of α-flu blocked the intra-rVTA (100 μM) caffeine reward. (b) In a separate group of animals treated with intra-rVTA (100 μM) caffeine, intra-NAc core (NAcC in graph) α-flu pretreatment had no effect on caffeine reward. (c) For animals treated with systemic caffeine (10 mg/kg i.p.), animals experienced significant aversions to caffeine regardless of pretreatment condition. (d) Animals treated with caffeine (10 mg/kg i.p.) exhibited caffeine aversions that could be blocked by pretreatment with α-flu in the NAc core. For each graph, bars represent the mean amount of time that animals spent in the drug-paired minus vehicle-paired sides of the testing apparatus ± *SEM*s. *$p < .05$

manifestation of caffeine reward, we injected α-flu into either the shell or core as a pretreatment, followed by an injection of caffeine into the rVTA. Animals treated with intra-rVTA caffeine (100 μM) experienced caffeine reward that could be blocked by α-flu pretreatment within the NAc shell (*t* test, $p = .0366$, $n = 15$) (Figure 6a). Conversely, this effect was not observed when α-flu was injected into the NAc core (*t* test, $p = .6240$, $n = 15$) as compared to vehicle pretreatment (Figure 6b). This suggests that the rVTA-NAc shell pathway is necessary for DA-dependent caffeine reward, but not the core.

3.6 | Systemic caffeine aversion is mediated through the NAc core, but not shell

Given the evidence showing that the NAc core is not necessary for caffeine reward in the conditioned place preference paradigm but is implicated in nicotine taste aversions (Sellings et al., 2008), we hypothesized that the core would mediate caffeine aversions. As higher doses of systemic caffeine produced conditioned place aversions that were blocked by systemic α-flu, rats were pretreated with injections of α-flu targeting the NAc core prior to place conditioning. α-flu blocked the aversions produced by 10 mg/kg i.p. of caffeine (*t* test, $p = .0145$, $n = 16$), indicating that the NAc core specifically mediates caffeine aversion in a DA-dependent manner (Figure 6d). Additionally, we tested whether the rVTA-NAc shell reward pathway influenced the NAc core-mediated aversive pathway. We did not observe a block of conditioned place aversions to systemic caffeine in rats pretreated with α-flu targeted to the NAc shell (*t* test, $p = .9077$, $n = 15$) (Figure 6c). As our earlier findings indicated that caffeine reward may be mediated by the rVTA-NAc shell pathway, and not the NAc core, the data in this section serve to double-dissociate functionally segregated caffeine reward and aversion pathways in the NAc shell and core, respectively.

4 | DISCUSSION

Assigning motivational valence to stimuli is a fundamental task for the mammalian brain. We have elicited separately conditioned caffeine reward through low-dose systemic injections and intra-rVTA caffeine, or conditioned caffeine aversions through higher dose systemic injections by performing in vivo administration of caffeine in a place conditioning paradigm. While our study aligns generally with the findings reported previously by us and by others (Brockwell, Eikelboom, & Beninger, 1991; Patkina & Zvartau, 1998; Sturgess et al., 2010), there are several inconsistencies and

that should be noted. For example, caffeine-conditioned place preferences have been observed from doses ranging from 0.32 to 10 mg/kg (Bedingfield et al., 1998), while others have observed no motivational effects of caffeine at 10 and 20 mg/kg (Poleszak & Malec, 2002). However, the differences in place conditioning method could account for these discrepancies. Some common differences include variation in experimental timelines and conditioning apparatuses (Bedingfield et al., 1998; Poleszak & Malec, 2002). More important, pharmacological blockade with dopamine receptor antagonists (non-selective DA, D1-like and D2-like) revealed that caffeine motivation is DA-dependent. Caffeine reward and aversion were blocked by α-flu in the NAc shell and core, respectively, suggesting that there are two separate and double-dissociated DA-dependent pathways.

Previous work in mice demonstrated that 10 and 30 mg/kg i.p. of caffeine produced aversions (Sturgess et al., 2010), which were recapitulated in rats. This aversion was dependent on DA activity in the NAc core, as both systemic and direct intra-NAc core injections of α-flu blocked aversions. Given that systemic caffeine should reach both NAc subregions, it is unclear why the NAc core-mediated aversion prevails at these higher caffeine doses. It may be that these doses reach higher effective concentrations in the VTA (higher than 100 μM of intra-rVTA caffeine) and overshadow the rewarding effects of rVTA activation, resulting in an overall aversion. This could be through a cVTA-mediated mechanism, which includes the tail of the VTA. The tail of the VTA is composed of a larger proportion of γ-aminobutyric acid (GABA) neurons than DA neurons and acts as a brake to the midbrain DA systems (Balcita-Pedicino et al., 2011; Bourdy & Barrot, 2012; Lammel et al., 2012; Sanchez-Catalan et al., 2017). Higher caffeine doses could preferentially activate this region, leading to a brake on the DA-mediated caffeine reward in the proposed rVTA-NAc shell pathway (Balcita-Pedicino et al., 2011; Bourdy & Barrot, 2012). Consistent with this notion, Kaufling and colleagues quantified the number of FosB/ΔFosB-positive nuclei (immediate early gene indicating cell activation) following caffeine exposure and found that doses equal or higher than 10 mg/kg i.p. induced a significant increase in recruited FosB/ΔFosB-positive nuclei in the tail of the VTA as compared to saline exposure (Kaufling et al., 2010). Given that the GABA-rich tail of the VTA provides inhibitory input to VTA DA cells (Matsui & Williams, 2011), this would also predict a lower activation of FosB/ΔFosB in the rVTA. Therefore, caffeine reward elicited at lower doses through the rVTA-NAc shell reward pathway would be inhibited when caffeine is systemically administered at high doses. However, at the highest caffeine dose of 60 mg/kg i.p., rats did not experience the expected higher aversion. This could be because the higher caffeine doses were more stressful to the animal. High doses of caffeine cause the body temperature of an animal to rise, which indicates physiological stress (Pechlivanova, Tchekalarova, Nikolov, & Yakimova, 2010). In response to stress, animals become anhedonic, reduce locomotor activity and reduce exploratory activity (Rygula et al., 2005). These behavioural changes that represent a loss of interest may interfere with learning about drug-place cues during conditioning. Another potential modulatory loop to consider at higher doses of systemic caffeine is that of the NAc projections back to the VTA. The GABAergic medium spiny neurons in the NAc appear to primarily project back to the non-dopaminergic neurons in the VTA (Xia et al., 2011; Yang et al., 2018). Local connections in the VTA may inhibit caffeine reward elicited by the rVTA-NAc shell pathway.

Caffeine reward was seen at a single low dose in a similar range (1.5 to 3.0 mg/kg) reported by others (Brockwell et al., 1991; Patkina & Zvartau, 1998). Further, caffeine reward was dependent on activity localized to the rostral VTA. There is ample evidence for heterogeneity along the VTA rostrocaudal axis from anatomical and behavioural studies (Ikemoto, 2007; Olson & Nestler, 2007; Olson et al., 2005; Yamaguchi, Sheen, & Morales, 2007). Our findings that caffeine can elicit reward only in the rostral VTA align closely with the work of Olson and colleagues who administered cocaine to the rostrocaudal subregions of the VTA and found that cocaine only in the rVTA could elicit place-conditioned reward (Olson et al., 2005). As caffeine reward was blocked by α-flu in the NAc shell, the rVTA DA neurons are the most likely candidates for mediating rewards. The functional distinction along the rostrocaudal axis could be explained if the rVTA DA neurons project more to the NAc shell and the cVTA neurons project more to the NAc core. A recent retrograde labelling study found that more cells from injections in the NAc shell than the core were positive for a DA marker (tyrosine hydroxylase, TH) in the rostral VTA – an anatomical VTA subregion, that along with the rostral parabrachial pigmentosus, comprises the rVTA defined in the present study (Rodriguez-Lopez, Clasca, & Prensa, 2017). Additionally, TH-positive (DA) neurons are present most highly in the parabrachial nucleus subdivision of the rat VTA (Nair-Roberts et al., 2008). However, further studies will be required to determine more quantitatively the strength of the specific connections from the rVTA to NAc shell versus core. Notably, the functional heterogeneity of the nucleus accumbens is arguably more well-defined than that of the VTA (Carlezon & Thomas, 2009; Di Chiara, 2002; Ikemoto & Panksepp, 1999; Jones, O'Dell, Marshall, & Wightman, 1996; Pontieri, Tanda, & Chiara, 1995; Solinas et al., 2002). For example, intravenous cocaine self-administration preferentially increased levels of DA in the NAc shell over the core (Pontieri et al., 1995). Also, DA receptor antagonism in the NAc core and shell has dissociable effects, where blocking D1-like receptors in the shell switch nicotine-dependent reward to aversion and blocking D2-like receptors in the

core in nicotine-deprived animals switch aversion to reward (Laviolette, Lauzon, Bishop, Sun, & Tan, 2008). This study presents additional evidence for the opposing roles of the NAc subregions in mediating drugs of abuse.

Over 89% of adults in the United States consume caffeine regularly (Fulgoni, Keast, & Lieberman, 2015). Though direct comparisons of caffeine consumption between people and rodents ought to be made with caution and with consideration of the higher metabolic functions in rodents, it is estimated that 1 cup of coffee is roughly equivalent to a dose of 2.3–5 mg/kg in rodents (Fredholm et al., 1999; Lao-Peregrin et al., 2016). Our data, then, represent a range of caffeine consumed by people of around 0.4–6 cups of coffee, and suggest that caffeine's motivational effects at these doses are mediated by mesolimbic dopaminergic pathways. Other psychoactive drugs such as ethanol and nicotine also appear to involve the mesolimbic system (Grieder et al., 2010; Ikemoto & Panksepp, 1999; Lammel, Ion, Roeper, & Malenka, 2011; Laviolette & van der Kooy, 2003; Ting-A-Kee et al., 2013; Wise & Morales, 2010). For example, reward is mediated by mesolimbic DA neurons when animals are given acute ethanol (Ting-A-Kee et al., 2013). However, when animals become chronically dependent on ethanol and are then deprived, further administration of ethanol is thought to be rewarding due to alleviation from the aversive feelings of deprivation. This process is thought to be mediated by the tegmental pedunculopontine nucleus (Ting-A-Kee et al., 2013). However, as this brain region is not involved in acute caffeine reward, further investigations are necessary to determine the effects of acute versus chronic caffeine.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

MY, RT and DV designed the experiments. DV provided the materials and facilities. MY, GM, RT, MC, IM, MB and TG performed the experiments. MY, GM, RT and MC analysed the data. MY and DV drafted the manuscript with revision made by the other authors.

DATA AVAILABILITY STATEMENT

Individual animal data are available on request from the corresponding author.

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