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RESEARCH REPORT

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Conditioning- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats

Morgane Colom[1,2,3] | **Igor Kraev**[1] | **Agata K. Stramek**[1] | **Iwona B. Loza**[1] | **Claire L. Rostron**[1] | **Christopher J. Heath**[1] | **Eleanor J. Dommett**[1,2] | **Bryan F. Singer**[1,4]

[1]School of Life, Health and Chemical Sciences, The Open University, Milton Keynes, UK

[2]King's College, Institute of Psychiatry, Psychology and Neuroscience, London, UK

[3]Department of Psychology, University of Cambridge, Cambridge, UK

[4]School of Psychology, Sussex Neuroscience, Sussex Addiction Research and Intervention Centre, University of Sussex, Brighton, UK

Correspondence
Bryan F. Singer, School of Psychology, University of Sussex, Brighton, UK.
Email: b.f.singer@sussex.ac.uk

Funding information
Faculty of Science, Technology, Engineering and Mathematics (STEM) at The Open University

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Abstract

For a subset of individuals known as sign-trackers, discrete Pavlovian cues associated with rewarding stimuli can acquire incentive properties and exert control over behaviour. Because responsiveness to cues is a feature of various neuropsychiatric conditions, rodent models of sign-tracking may prove useful for exploring the neurobiology of individual variation in psychiatric vulnerabilities. Converging evidence points towards the involvement of dopaminergic neurotransmission in the nucleus accumbens core (NAc) in the development of sign-tracking, yet whether this phenotype is associated with specific accumbal postsynaptic properties is unknown. Here, we examined dendritic spine structural organisation, as well as presynaptic and postsynaptic markers of activity, in the NAc core of male and female rats following a Pavlovian-conditioned approach procedure. In contrast to our prediction that cue re-exposure would increase spine density, experiencing the discrete lever-cue without reward delivery resulted in lower spine density than control rats for which the lever was unpaired with reward during training; this effect was tempered in the most robust sign-trackers. Interestingly, this same behavioural test (lever presentation without reward) resulted in increased levels of a marker of presynaptic activity (synaptophysin), and this effect was greatest in female rats. Whilst some behavioural differences were observed in females during initial Pavlovian training, final conditioning scores did not differ from males and were unaffected by the oestrous cycle. This work provides novel insights into how conditioning impacts the neuronal plasticity of the NAc core, whilst highlighting the importance of studying the behaviour and neurobiology of both male and female rats.

Abbreviations: CR, conditioned response; CS, conditioned stimulus; GT/GTs, goal-tracking/goal-trackers; LTP, long-term potentiation; MSNs, medium spiny neurons; NAc, nucleus accumbens; PB, phosphate buffer; PBS, phosphate buffer saline; PCA, Pavlovian conditioned approach; ST/STs, sign-tracking/sign-trackers; US, unconditioned stimulus.

KEYWORDS
dendritic spines, motivation, nucleus accumbens, rat, sign-tracking, synaptic plasticity

1 | INTRODUCTION

Environmental stimuli can provide valuable information by signalling the availability of resources and rewards. Such rewards can be imbued with appetitive and motivational properties which prompt people and animals to actively seek and approach them (Berridge & Robinson, 2003). Incentive characteristics may be transferred to discrete reward-predicting cues, transforming them into attractive, desirable stimuli capable of invigorating behaviour; however, individuals vary in the magnitude of this transition (Cardinal et al., 2002; Robinson & Flagel, 2009). The emergence of distinct cue-directed conditioned responses (CR) is often studied using a rodent Pavlovian conditioned approach (PCA) procedure in which a lever (conditioned stimulus; CS) is repeatedly paired with the delivery of food pellets (unconditioned stimulus; US). As animals learn that the lever is predictive of the reward availability, goal-trackers (GTs) acknowledge the cue and approach the reward location, whereas sign-trackers (STs) develop a conditioned approach response towards the CS, only moving to the food cup after retraction of the lever (Flagel et al., 2009). Other individuals exhibit an intermediate response and oscillate between both strategies. Whilst equally predictive in both ST and GT phenotypes, a discrete CS only possesses incentive and motivational value for STs (Flagel et al., 2009).

Mesolimbic dopamine neurotransmission, particularly in the core subregion of the nucleus accumbens (NAc), has been implicated in the degree to which Pavlovian cues become incentive stimuli. Reward-associated CSs induce a greater increase of dopamine release in this area in STs compared to GTs (Aitken et al., 2016; Flagel et al., 2011; Singer, Bryan, et al., 2016). The disruption of dopamine signalling in the NAc core specifically impairs the acquisition and the performance of conditioned responses in STs (Blaiss & Janak, 2009; Fraser & Janak, 2017; Saunders & Robinson, 2012), whilst stimulating dopaminergic neurotransmission preferentially enhances ST's approach towards the CS (Singer, Guptaroy, et al., 2016). Furthermore, simultaneous optogenetic stimulation of dopamine release in the NAc core, along with cue presentation appears sufficient to confer incentive properties to the CS (Saunders et al., 2018).

Inputs from dopaminergic neurons typically synapse onto dendritic spines of GABAergic medium spiny neurons (MSNs) in the NAc; therefore, spines are major targets of activity-dependent neuroplasticity associated with appetitive learning (Geinisman et al., 2001; Leuner et al., 2003; Matsuzaki et al., 2004; Singer et al., 2009; Singer, Bubula, et al., 2016). Consequently, the structural organisation of dendritic spines in the NAc is modulated by dopaminergic signalling (Yao et al., 2008). Dopamine depletion or blockade results in a decrease in spine length and density (Fasano et al., 2013), whereas raising dopamine levels leads to an increase in spine density, branching and length in MSNs (Fasano et al., 2013; Meredith et al., 1995). Environmental stimuli paired with rewards can induce morphological reorganisations of dendritic spines in the NAc (Singer, Bubula, et al., 2016).

When reward-associated cues are attributed with disproportionate motivational/emotional salience, they can contribute to specific sets of symptoms within neuropsychiatric conditions such as excessive drug use and gambling-like behaviours – but also trauma-related responses (Anselme & Robinson, 2020; Casada et al., 1998; Felix & Flagel, 2024; Morrow et al., 2011; Robinson et al., 2016; Saunders & Robinson, 2010). Additionally, cue-induced reinstatement of drug-seeking as well as repeated drug use can result in drug-specific postsynaptic alterations in the NAc core (Gipson et al., 2013; Kalivas & O'Brien, 2008). Characterising the neurobiological basis of individual variation in cue-motivated behaviour may, therefore, have translational clinical relevance, helping us understand why some individuals are susceptible to neuropsychiatric conditions that involve learning and motivation. As sign- and goal-tracking phenotypes are associated with distinct profiles of dopaminergic neurotransmission, it is conceivable that changes in dendritic spines in the NAc core might reflect the incentive value of reward-predictive cues, and that individual variation in cue-induced motivation may be attributable to differences in postsynaptic plasticity. Here, we assessed these hypotheses regarding cue-induced synaptic changes in the NAc core of rats classified as STs. Variations in Pavlovian conditioned responses were compared to dendritic spine measurements, as well as markers of presynaptic and postsynaptic activity: synaptophysin, one of the most abundant proteins expressed in synaptic vesicles and commonly used as a presynaptic marker (Grijalva et al., 2021; Ota et al., 2010); and homer1, a component of the postsynaptic density which is also altered by learning-induced LTP (Clifton et al., 2019; Petrovich

et al., 2005). Overall, the results were novel and surprising, suggesting that our hypothesis about postsynaptic changes reflecting incentive value may not be entirely accurate. Instead, we observed how synapses in the NAc core changed in response to reward omission, and that the most robust STs were resistant to such plasticity.

Neurobiological and symptomatic sexual dimorphisms can be observed in conditions involving a dysfunctional salience of environmental cues (Becker & Chartoff, 2019; Fattore et al., 2014; Kokane & Perrotti, 2020; Pooley et al., 2018), which is why sex must remain a critical variable to consider. Although a few rodent sign-tracking studies have included both males and females, the results were heterogeneous (Dickson et al., 2015; Fuentes et al., 2018; Hughson et al., 2019; King et al., 2016; Kucinski et al., 2018; Madayag et al., 2017; Pitchers et al., 2015). Thus, both male and female rats were examined and compared in the present work. Moreover, as sex hormones fluctuate across the rat's oestrous cycle and produce behavioural and physiological changes (Becker et al., 2005), we performed a secondary analysis of behavioural data describing the influence of the oestrous cycle on conditioned behaviour.

2 | MATERIALS & METHODS

2.1 | Animals

A total of 45 male and 48 female Lister Hooded rats (outbred; Charles River, Kent, UK; RRID: SCR_003792) aged from 4 to 6 weeks upon arrival were housed in same-sex groups of 3 and kept on a reverse 12 h-light, 12 h-dark cycle (dark at 08:00 AM). Testing was conducted during the dark phase. All cages (GPR2) were kept in the same ventilated cabinet (Scantainer) in which the ambient temperature was maintained at 21–23°C. Animals had ad libitum access to water and food (RM3 diet, Special Diet Services, Essex, UK) throughout the experiment, and cages were supplied with environmental enrichment in the form of Aspen wooden chew blocks, cardboard tunnels, as well as bedding and nesting material. Rats were left to acclimatise for 6 weeks until they reached adulthood to maximise the familiarisation with the facilities and experimenters, as well as to ensure the consistency of the oestrous cycle in females (age of testing: 11 to 14 weeks; 331-513 g for males, 197-369 g for females). All animal procedures were reviewed and approved by the Animal Welfare Ethical Review Body (AWERB) and were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 and EU Directive 86/609/EEC (UK Home Office Project Licence PABC1F4D1).

2.2 | Oestrous cycle monitoring

Female behaviour and physiology fluctuate throughout the 4 or 5 days of the rat ovarian cycle (Becker et al., 2005). Due to limited resources and to ensure consistency of testing parameters, neurobiological assessments of females occurred during diestrus, as there may be less variation in dendritic spine properties between males and females in this phase (Woolley et al., 1990). Because cytology, which is used as a gold standard to estimate the oestrous stage, is an invasive procedure, cycles of female rats were instead monitored using the lordosis behaviour displayed during the peri-ovulatory phase to recognise the proestrus stage and consequently predict the other phases in a non-invasive manner (Stramek et al., 2019; Video S1). The oestrous cycle was considered established after two weeks of regularity, and the stage was confirmed by visually examining the vaginal smear before intracardiac perfusions whilst animals were under anaesthesia (Becker et al., 2005; Stramek et al., 2019).

2.3 | Apparatus

Conditioning took place in 29.53 × 23.5 × 27.31 cm modular test chambers composed of a stainless-steel grid floor and a retractable lever located on the left- or right-hand side of a food magazine, in sound-attenuating compartments (Med Associates, Inc.; St Albans, VT, USA; RRID: SCR_021938). A house light remained illuminated during sessions. Test chambers were attributed to male or female rats to prevent odour contamination and were cleaned with 70% ethanol between each animal.

2.4 | Pavlovian conditioned approach

2.4.1 | Pre-training

On day 1, Animals were familiarised with the food pellets used in the behavioural procedure (AIN-76A Rodent Tablet 45 mg, TestDiet) by placing a handful in home cages two days before the beginning of training (Figure 1). On the day prior to training (day 2), rats were habituated to the apparatus in a single pre-training session during which 25 pellets were delivered on a variable time schedule (0–45 seconds range) after 5 minutes of

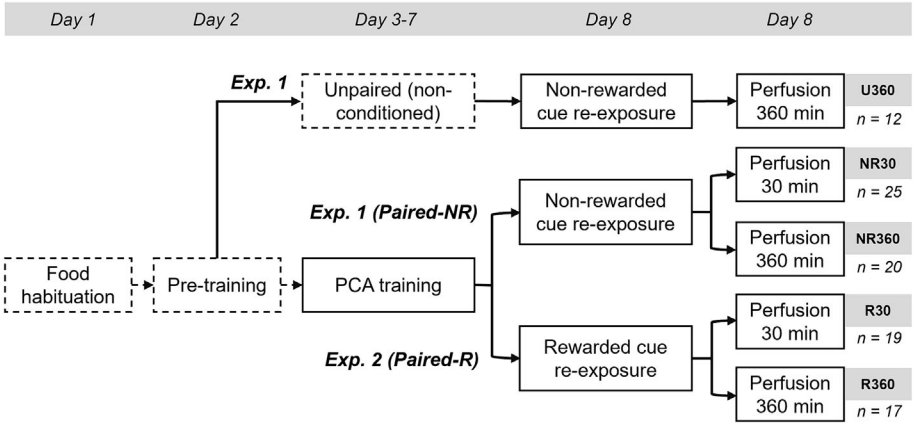

FIGURE 1 Schematic illustration of the experimental timeline. Animals were trained in a paired or unpaired fashion, before being re-exposed to the lever in the presence (Exp. 1) or absence (Exp. 2) of the reward. Rats were perfused at two-time points after the re-exposure session. *NR30*: paired rats perfused 30 minutes after a non-rewarded cue re-exposure. *NR360*: paired rats perfused 360 minutes after a non-rewarded cue re-exposure. *R30*: paired rats perfused 30 minutes after a rewarded cue re-exposure. *R360*: paired rats perfused 360 minutes after a rewarded cue re-exposure. *U360*: unpaired rats perfused 360 minutes after re-exposure to the lever.

acclimatisation in the dark. Rats were weighed before each daily session to monitor their welfare.

2.4.2 | Cue-paired training

Paired (i.e., conditioned) animals (Experiment 1 and 2; females $n = 42$, males $n = 39$) were trained in a Pavlovian setting for five consecutive days (Figure 1; days 3 to 7). During each of the 25 trials composing a session, the lever (CS +) extended and illuminated for 8 seconds, and its retraction was immediately followed by the delivery of one food pellet (US) in the magazine. Cue-reward pairings occurred on a variable time schedule (30–150 seconds range).

2.4.3 | Unpaired training

A distinct cohort of Unpaired rats (Experiment 1; females $n = 6$, males $n = 6$) was exposed to the same number of lever presentations and pellet deliveries as Paired rats (Figure 1; days 3 to 7), however, lever presentation was not temporally associated with reward delivery. Thus, only behaviour observed in Paired animals emerged from the association between the CS+ and the US.

2.5 | Cue re-exposure

2.5.1 | Experiment 1

On a final test day (day 8), which occurred from 2 to 4 days after training to target the diestrus stage of female rats, a subset of Paired rats (group **NR**: females $n = 24$, males $n = 21$) and all Unpaired rats (group **U**: females $n = 6$, males $n = 6$) were placed in the same behavioural chambers for 5 minutes in order to induce contextual extinction, and were subsequently re-exposed to the CS in the absence of reward to isolate the incentive from the predictive properties of the cue (Figure 1; Flagel et al., 2011; Yager et al., 2015). The lever was presented 10 times for 4 seconds on a variable time 90-second schedule (30–150 seconds); no food pellets were delivered in the magazine following lever retraction.

2.5.2 | Experiment 2

Based on the results of Experiment 1, Experiment 2 was designed to assess whether delivering the reward in conjunction with cue exposure impacted synaptic plasticity. Accordingly, two to four days after training (day 8), a different cohort of Paired rats (group **R**: females $n = 18$, males $n = 18$) was subjected to a single test session starting with a contextual extinction of 5 minutes, after which the CS was extended 10 times for 4 seconds on a variable time 90-seconds schedule (30–150 seconds) and was immediately followed by the delivery of a food pellet (Figure 1).

2.6 | Timing of synaptic assessment after cue re-exposure

In order to separate immediate, baseline plasticity from later synaptic changes induced by the predictive cue, brains were collected at two different time points after the end of the lever re-exposure session (day 8). A subset of non-rewarded Paired animals (Experiment 1, group **NR30**:

females $n = 14$, males $n = 11$) and rewarded Paired animals (Experiment 2, group **R30**: females $n = 9$, males $n = 10$) were immediately anaesthetised at the end of the test session and consequently perfused 30 minutes after (Figure 1). The remaining non-rewarded Paired rats (Experiment 1, **NR360**; females $n = 10$, males $n = 10$) and rewarded Paired rats (Experiment 2, group **R360**: females $n = 9$, males $n = 8$) were returned to home cages after the test session and left undisturbed for 360 minutes (6 hours) before perfusion to ensure that potential morphological and synaptic changes had time to take place (Figure 1; De Roo et al., 2008). All Unpaired animals were perfused 360 minutes (6 hours) after lever re-exposure (Experiment 1, group **U360**; Figure 1). Accordingly, either 30 minutes or 360 minutes after the lever re-exposure session, animals were deeply anaesthetised with isoflurane 5% (Zoetis, US) and were subsequently injected with an intraperitoneal overdose of pentobarbitone sodium (0.6–0.8 ml/kg; Animalcare, UK). Rats were perfused intracardially with 0.1 M phosphate-buffered saline (PBS; pH = 7.4) and heparin, then with 3% paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.1 M phosphate buffer (PB; pH = 7.4).

2.7 | Golgi-cox staining

2.7.1 | Tissue preparation

Following fixation, coronal brain sections (100 μm) which included the NAc core were collected (Figure 2a; Leica VT1000 vibratome; RRID: SCR_016495) and stored in 2.5% glutaraldehyde in 0.1 M PB at +4°C until Golgi impregnation staining on single section (Gabbott & Somogyi, 1984). Three to six sections per animal were rinsed in 0.1 M PB, processed in 1% osmium tetroxide and 0.2 M PB for 40 minutes, and subsequently left overnight at room temperature in 3.5% potassium dichromate in distilled water. Brain sections were then mounted between two glass slides to form a 'sandwich' and submerged in a vertical position into a 1.5% silver nitrate solution for a maximum of 4 hours. When first signs of staining were detected under the microscope, the 'sandwich' was disassembled and the reaction was stopped with glycerol, then sections were covered with cover glasses and left overnight at +4°C. The following day, brain sections were dehydrated using ascending concentrations of ethanol (50%, 70%, 90%, 100%), then cleared in xylene, and finally mounted onto glass slides and coverslipped with DPX.

2.7.2 | Anatomical measures

Image stacks were taken using a light microscope (Nikon Eclipse 80i upright; 100x objective; RRID: SCR_015572) through the z-axis in the NAc core of stained brain slices, identified as the area within 250 μm around the anterior commissure (Figure 2a,b). Proximal dendrites from at least three different neurons and longer than 30 μm were manually outlined, and dendritic spines were delineated by individually adjusting the size of the head and attaching it

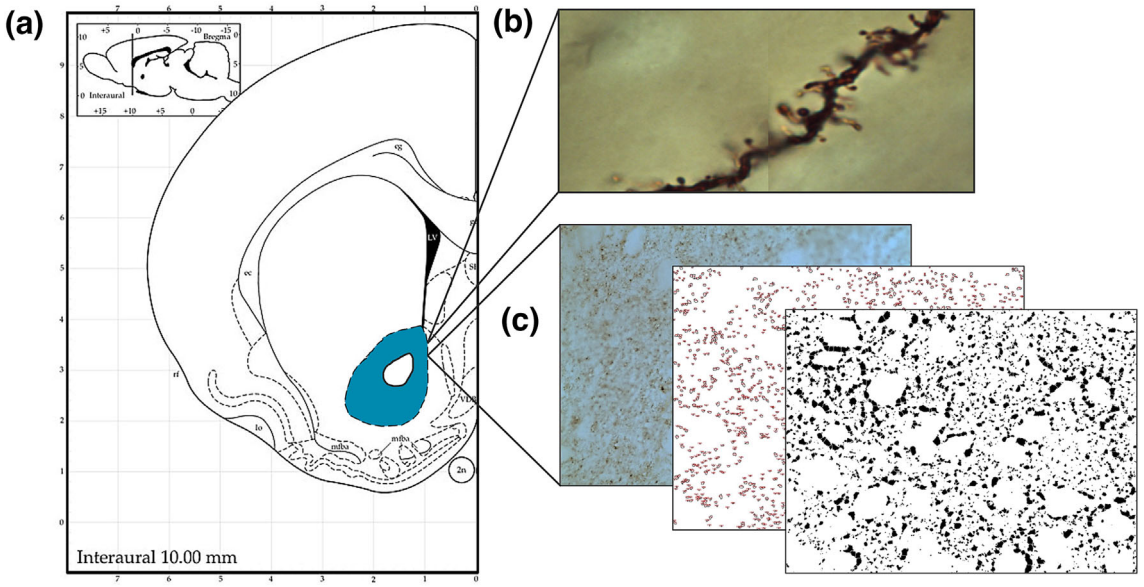

FIGURE 2 Dendritic spines and presynaptic markers in the NAc core. (a) Coronal view of the NAc core (*blue*) in blue in the ventral striatum. *Image modified from the atlas of Paxinos and Watson, 2006*. (b) Photograph of Golgi-stained spines on a NAc core dendrite. (c) From left to right: Synaptophysin stained tissue in the NAc core. Images are merged through the z-axis and thresholded, puncta exceeding 200 μm are excluded, and the remaining puncta are quantified.

to each dendrite whilst keeping all parameters constant throughout images (Neurolucida neuron tracing software, MBF Bioscience, Williston, VT, USA; RRID: SCR_001775). Quantitative analyses subsequently determined spine density per dendrite length, spine length and spine head diameter (Neurolucida Explorer, MBF Bioscience, Williston, VT, USA; RRID: SCR_ 017348).

2.8 | Synaptophysin and homer1 immunohistochemistry

2.8.1 | Tissue preparation

Concurrently to slicing sections for the Golgi-impregnation staining, coronal slices of the NAc core were collected (50 μm; Leica VT1000 vibratome; RRID: SCR_016495), stored in a cryoprotectant storage solution made from sucrose and ethylene glycol in PB 0.1 M, and kept at −20°C until immunostaining. Two to three sections per animal were left to warm at ambient temperature, then rinsed with 0.1 M PB and left in 1% sodium borohydride for 30 minutes, after which the resulting effervescence was rinsed out with 0.1 M PB. Peroxidase was blocked by placing the slices in 10% methanol and 3% hydrogen peroxide in 0.1 M PB for 5 minutes. Sections were transferred in 5% skimmed milk in 0.1 M PB for 15 minutes to block endogenous biotin, before being rinsed with 0.1 M PB once more. Brain slices were then moved to a blocking buffer (0.5% Bovine Serum Albumin and 0.3% Triton X-100 in 0.1 M PB) for 30 minutes and subsequently incubated either in 1:100 polyclonal rabbit anti-homer1 antibody (Synaptic Systems, Germany), or in 1:1000 monoclonal mouse anti-synaptophysin antibody (Synaptic Systems, Germany; Cat# 101011C3, RRID: AB_887822) for 36 h on a gentle shaker at +4°C. Next, sections were rinsed in 0.1 M PB, incubated for a further two hours in either 1:200 donkey anti-rabbit biotinylated antibody (Jackson ImmunoResearch, USA; Cat# 711–065-152, RRID: AB_2340593) or 1:200 donkey anti-mouse biotinylated antibody (Jackson ImmunoResearch, USA; Cat# 715-065-150, RRID: AB_2307438), rinsed with 0.1 M PB, and moved in ABC peroxidase (two drops of solution A and B for every 10 ml of 0.1% Bovine Serum Albumin in 0.1 M PB) for 30 minutes. DAB in 30% hydrogen peroxide and 0.1 M PB was used to develop staining and was subsequently rinsed with 0.1 M PB, after which sections were left to dry on gelatine-coated glass slides for 48 hours. Lastly, sections were dehydrated in ascending concentrations of ethanol (30%, 50%, 70%, 90%, 100%), cleared in xylene and coverslipped using DPX.

2.8.2 | Anatomical measures

For each animal, image stacks through the z-axis were taken from 10 random sites within the NAc core, 250 μm around the anterior commissure (Olympus BX53, 100x objective; RRID: SCR_022568). Using ImageJ (Schneider et al., 2012; RRID: SCR_003070), and whilst keeping all parameters constant throughout samples, image stacks were then merged, the background was subtracted and the image thresholded (Figure 2c). Merged puncta smaller than 10 μm and larger than 200 μm were removed to exclude potential artefacts (Figure 2c). Finally, the number of puncta, the average size of puncta, as well as the density of puncta per area were determined using the 'analyse particles' function.

2.9 | Pavlovian conditioned approach quantification

Test chambers were fitted with sensors recording head entries into the food cup and lever deflections. On days 4 and 5 of training, once conditioned responses were deemed established, the latency and probability of contacting the lever and the food magazine during lever presentation, as well as the number of contacts with the lever and the food magazine during lever presentation, were extracted (Med Associates software, Inc.; St Albans, VT, USA; RRID: SCR_012156). These measures were then used to calculate a PCA index score by averaging the response bias [(lever presses – food cup entries)/(lever presses + food cup entries)]; the probability difference [p (lever presses) – p (food cup entries)]; and the latency score [(average food cup entry latency – average lever press latency)/8] (Meyer et al., 2012). The final PCA score extended from −1.0 to +1.0, with the lowest score reflecting animals producing a GT-conditioned response on every trial, and the highest score indicating a ST-conditioned response on every trial. Animals with a PCA score ranging from −1.0 to −0.4 were classified as GTs, whereas individuals with a PCA score ranging from +0.4 to +1.0 were categorised as STs. Scores between −0.39 and +0.39 suggested intermediate conditioned responses. Due to the low number of GTs ($n = 4$ in Experiment 1, $n = 4$ in Experiment 2), we did not analyse STs vs. GTs, nor intermediates. Instead, we assessed neurobiology across groups of STs (Figure 1; NR30, NR360, R30, R360, where the numbers refer to the lapse of time between the test session and the perfusion, i.e., 30 or 360 minutes later), and versus Unpaired animals (U360).

2.10 | Statistical analysis

GraphPad Prism (versions 8, 9 and 10; GraphPad Software Inc.; San Diego, CA, USA; RRID: SCR_002798) was used for ANOVAs, correlations and independent group comparisons. Chi-square analyses were conducted in SPSS (versions 27 and 28; IBM Corp.; Chicago, IL, USA; RRID: SCR_019096). All group comparison results are presented as mean + SEM. Statistical significance was set at $\alpha = 0.05$. Measures were all checked for normality using the Shapiro–Wilk test and non-parametric tests were used when appropriate.

The initial training of Paired and Unpaired groups (e.g., the interaction with the lever and the food cup) was analysed using two-way repeated measures ANOVAs, with sex as between-subject factor and session as within-subject factor. The phenotypic repartition of males and females during training was compared using the Chi-square test of independence. Two-way ANOVAs were used to compare the neurobiology of Paired-NR360, Paired-NR30, Paired-R360, Paired-R30, and Unpaired rats (e.g., dendritic spines, synaptophysin staining and homer1 staining), as well as sex differences during the re-exposure session in Paired groups. Significant interactions or main effects were followed by Šídák post-hoc tests to observe the specific effect of sex on plasticity. The interaction with the lever during the test session between NR and R groups, along with lever-directed and food cup-directed interactions between male and female Unpaired rats, were investigated using either parametric independent *t*-tests or non-parametric Mann–Whitney *U* tests. The evolution of conditioned responses across trials during the re-exposure session of Experiments 1 and 2 was examined using the non-parametric Friedman test for repeated measures, which was followed by a Dunn's multiple comparisons test when a significant difference was found. All correlations were analysed using Pearson's correlations when the dataset met normality assumptions or Spearman's correlations when it did not. The effect of the oestrous cycle on behaviour was assessed with two-way ANOVAs because animals in each female group were different depending on the training session (see Table S1) – thus repeated measures were absent, apart from the male group. When significant interactions or main effects were detected, the three groups were compared using Tukey post-hoc tests. Violations of statistical assumption were dealt with using log10 transformations.

One Unpaired female was identified as an outlier using the ROUT method and was removed from subsequent neurobiological analyses. One female ST from the NR30 group and one male ST from the NR360 group were excluded from the dendritic spine and synaptophysin analyses, respectively, due to damaged brain slices.

3 | RESULTS

3.1 | Effect of sex and learning on behavioural phenotypes

Paired animals were first trained in a PCA procedure, and their behavioural bias towards the CS and towards the US location was monitored. A strong bias towards sign-tracking was found in male and female rats of Experiment 1 (Figure S1) and Experiment 2 (Figure S2). When combining all STs from the NR (Exp 1) and R (Exp 2) Paired groups (Figure 3a, *full lines*), two-way repeated measures ANOVAs with sex as a between-subjects factor and session as a within-subject factor showed no sex difference in lever contacts (Figure 3a-1; $F_{1,\ 59} = 0.5551$, $p = 0.4592$), lever probability (Figure 3a-2; $F_{1,\ 59} = 0.1720$, $p = 0.6799$) or lever latency (Figure 3a-3; $F_{1,\ 59} = 1.169$, $p = 0.2839$). However, female STs appeared to interact with the food cup more than males. Indeed, a main effect of sex and a sex x session interaction was found in STs for the number of contacts with the food cup (Figure 3a-4; effect of sex: $F_{1,\ 59} = 4.837$, $p = 0.0318$; interaction: $F_{4,\ 236} = 6.547$, $p < 0.0001$) and the latency to first contact the food cup (Figure 3a-6; effect of sex: $F_{1,\ 59} = 8.447$, $p = 0.0051$; interaction: $F_{4,\ 236} = 6.533$, $p < 0.0001$). Another two-way repeated measures ANOVA revealed a sex x session interaction in the probability to contact the food cup during lever presentation (Figure 3a-5; $F_{4,\ 236} = 5.270$, $p = 0.0004$).

For the control Unpaired animals, lever presentation and delivery of food pellets were not temporally associated. In accordance with the previous literature (Flagel et al., 2007 and 2011; Lomanowska et al., 2011; Meyer et al., 2012; Robinson & Flagel, 2009; Saunders et al., 2018; Singer, Bryan, et al., 2016; Yager & Robinson, 2013), contrary to STs (Figure 3a) and GTs (data not shown), Unpaired rats neither learnt nor developed a conditioned response to the discrete stimuli, as evidenced by the stable interaction with the lever and the food magazine across sessions (Figure 3a, *dotted lines*). Unpaired males and females exhibited equivalent lever-directed behaviours (Figure 3a). However, like the conditioned rats, females appeared to interact more with the food cup than males; a two-way repeated measures ANOVA revealed an effect of sex in food cup contacts (Figure 3a-4; $F_{1,\ 9} = 49.75$, $p < 0.0001$), latency (Figure 3a-6; $F_{1,\ 9} = 9.824$, $p = 0.0120$) and probability (Figure 3a-5; $F_{1,\ 9} = 18.29$, $p = 0.0910$) of contacts during CS presentation.

The number of visits to the food cup outside of lever presentation is sometimes considered an indication of the rate of learning in that it should decrease as the animal learns that the US is delivered only after lever retraction

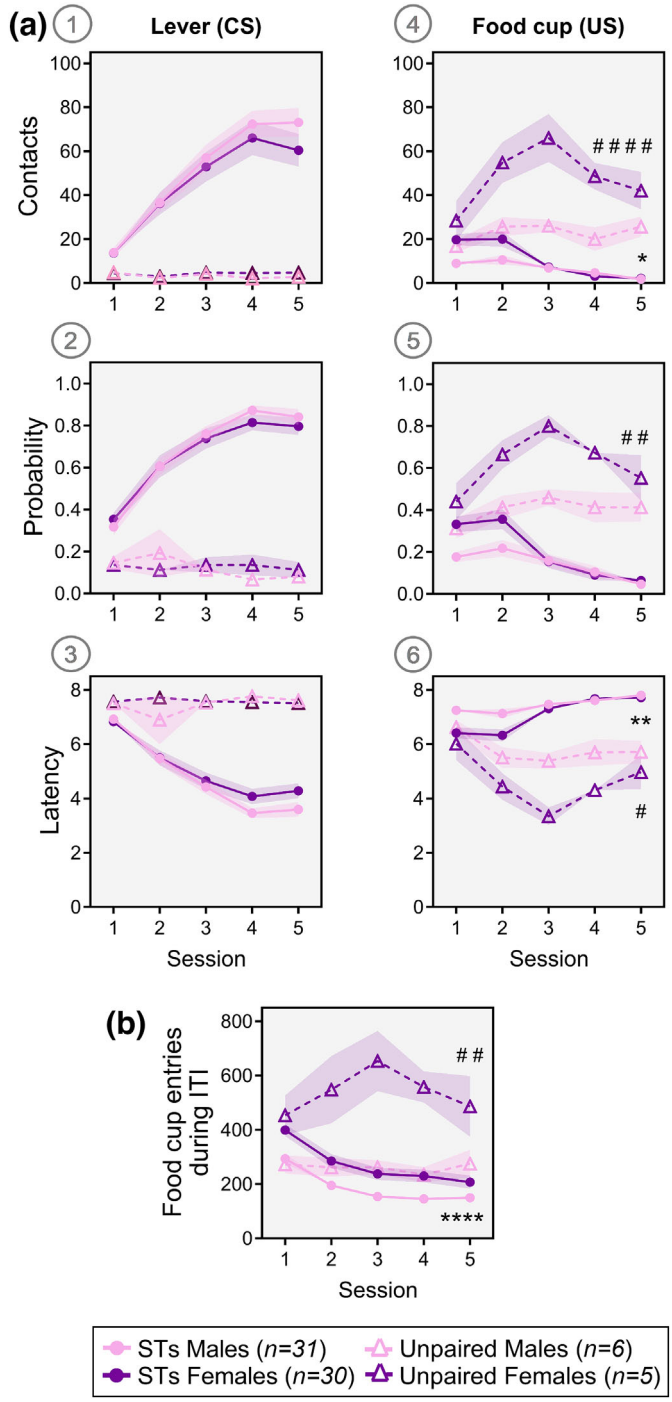

FIGURE 3 Interaction with the CS+ and the US during training. (a) *Full lines*: sex comparison of the total number of contacts to the lever [1] or the food cup [4] during the lever extension, the probability to contact the lever [2] or the food cup [5] during the lever extension, and the latency to first contact the lever [3] or the food cup [6] during the lever extension, for each session, between male and female STs (paired-R, re-exposed to both the lever and the reward, and paired-NR, re-exposed only to the lever, combined). *Dotted lines*: comparison between male and female unpaired rats. (b) *Full lines*: sex comparison of the total number of food cup entries during the inter-trial interval for each session between male and female STs, paired-R and paired-NR groups combined. *Dotted lines*: comparison between male and female unpaired rats. (paired: * $p \leq 0.05$, ** $p \leq 0.01$, **** $p < 0.0001$; unpaired: # $p \leq 0.05$, ## $p \leq 0.01$; #### $p < 0.0001$).

(Flagel et al., 2007), but can also suggest activity levels (Horner et al., 2013) or can alternatively represent a specific aspect of complex appetitive conditioned responses. Figure 3b shows the number of visits to the food cup in all STs (NR and R groups) and in Unpaired male and female rats across training sessions. Conditioned female

rats (STs from Experiments 1 and 2) were more likely to enter the food cup during inter-trial intervals than males, as demonstrated by a two-way repeated measures ANOVA with sex as between-subjects factors and session as a within-subject factor (Figure 3b; effect of sex: $F_{1,\ 59} = 17.82$, $p < 0.0001$). Similarly, Unpaired females visited the food cup significantly more than males (effect of sex: $F_{1,\ 9} = 21.20$, $p = 0.0013$). All animals behaved in a comparable way during the last training session regardless of their sex.

3.2 | Effect of conditioned behaviour on synaptic plasticity in male and female rats

Two to four days after training, animals from Experiment 1 (Paired-NR group) undertook a single session during which they were re-exposed specifically to the CS. In an attempt to prevent extinction, the lever was extended for only 4 seconds and for a total of 10 times (instead of 8 seconds and 25 times during conditioning). Apart from a non-statistically significant tendency for male STs to contact the lever faster than female STs (Figure S3.A-2; effect of sex: $F_{1,\ 57} = 3.885$, $p = 0.0536$; Šídák, $p = 0.0736$), all STs from the NR group contacted the lever (Figure S3.A-1; effect of sex: $F_{1,\ 57} = 2.438$, $p = 0.1240$), entered the food cup during CS presentation (Figure S3.B; $F_{1,\ 57} = 2.869$) and approached the food cup ($F_{1,\ 57} = 1.547$, $p = 0.2187$) at a similar rate. Unpaired female rats entered the food cup more than Unpaired males during the lever re-exposure session (Figure S3.D-1; $t = 3.300$, $df = 9$, $p = 0.0098$), but did not differ in their interaction with the lever (Figure S3.C-1; unpaired t-test: $t < 0.8435$, $df = 9$, $p > 0.4208$) or latency to approach the food cup (Figure S3.D-2; $t = 1.403$, $df = 9$, $p = 0.1940$).

A Friedman test for repeated measures revealed no difference in lever contacts (Figure SA4.A; $\chi^2 = 5.480$, $df = 9$, $p = 0.7906$) or latency to approach the lever ($\chi^2 = 12.69$, $df = 9$, $p = 0.1772$) across the 10 trials of the re-exposure session for NR360 animals, on which subsequent analyses will focus. A positive relationship was found in Paired animals between lever presses and latency during the last training session and the test session (Figure S4.B; all Pearson $r < 0.5447$, all $p > 0.0001$). It should be noted that NR STs' number of food cup entries was not comparable between the re-exposure session and the end of the training (Figure S6.A; Spearman $r = 0.1833$, $p = 0.2281$), perhaps due to the absence of reward delivery following the cue. Nonetheless, because histological analyses focussed on STs, the strong correlation between cue-directed CRs expressed during both sessions enabled us to use the former reliably to classify our animals into groups and perform our subsequent analyses.

Either 30 minutes (NR30) or 360 minutes (NR360) following the re-exposure session, neurons from the NAc core of unrewarded Paired and Unpaired animals were stained using 'single' section Golgi-impregnation staining. Dendritic spines were reconstructed, and spine density per dendrite length, spine head diameter and spine length were quantified. A total of 259 (Paired) and 65 (Unpaired) proximal dendrites from at least three different neurons taken from random locations in the NAc core were reconstructed (4 to 7 per rat; average diameter = 0.97 μm; length ranging from 31.9 to 181.1 μm, average length = 75.35 μm). Synaptophysin and homer1 puncta were quantified as a measure of pre- and postsynaptic activity, respectively. Apart from spine length, which was found to be shorter in STs perfused 360 minutes after CS re-exposure (NR360) compared to the NR30 group (Figure S5.A-3; effect of time: $F_{1,\ 30} = 5.256$, $p = 0.0291$), rats did not differ in their spine structural organisation (Figure S5.A; effect of time: all $F_{1,\ 29} < 1.056$, all $p > 0.3125$), synaptophysin staining (Figure S5.B; effect of time: all $F_{1,\ 30} < 1.349$, all $p > 0.3125$) or homer1 staining (Figure S5.C; effect of time: all $F_{1,\ 30} < 0.07838$, all $p > 0.7814$) in the NAc core. Therefore, only the NR360 and U360 groups were examined further.

Paired STs perfused 360 minutes after the non-rewarded cue re-exposure (NR360) were compared to the Unpaired group who did not undergo Pavlovian conditioning (Figure 4). A two-way ANOVA revealed an effect of the training wherein animals who learnt a Pavlovian association had a significantly smaller density of spines in the NAc core (Figure 4a-1; $F_{1,\ 22} = 16.12$, $p = 0.0006$). No difference was observed between both groups in spine length (Figure 4a-3; $F_{1,\ 22} = 1.103$, $p = 0.3050$) or average spine head diameter (Figure 4a-2; $F_{1,\ 22} = 0.5787$, $p = 0.4549$), and no sex difference was found.

The comparison of synaptophysin staining between STs of the NR360 group and Unpaired animals revealed sex and training differences (Figure 4b). More specifically, STs had significantly more synaptophysin puncta (Figure 4b-1; effect of training: $F_{1,\ 22} = 24.21$, $p < 0.0001$) and a greater density of puncta (Figure 4b-3; $F_{1,\ 22} = 24.20$, $p < 0.0001$) than Unpaired animals. The size of synaptophysin puncta was similar between both groups. Synaptophysin puncta of female STs were more numerous (Figure 4b-1; effect of sex: $F_{1,\ 22} = 10.49$, $p = 0.0038$; Šídák, $p = 0.0065$) and had higher density (Figure 4b-3; effect of sex: $F_{1,\ 22} = 10.49$, $p = 0.0038$; Šídák, $p = 0.0033$) than those of male STs. Within the NR360 group, no relationship was found between synaptophysin staining and the latency to first approach the CS (Figure S7.A; all Pearson $r < 0.1151$, all $p > 0.6492$) or the number of lever presses (Figure S7.C; all Pearson

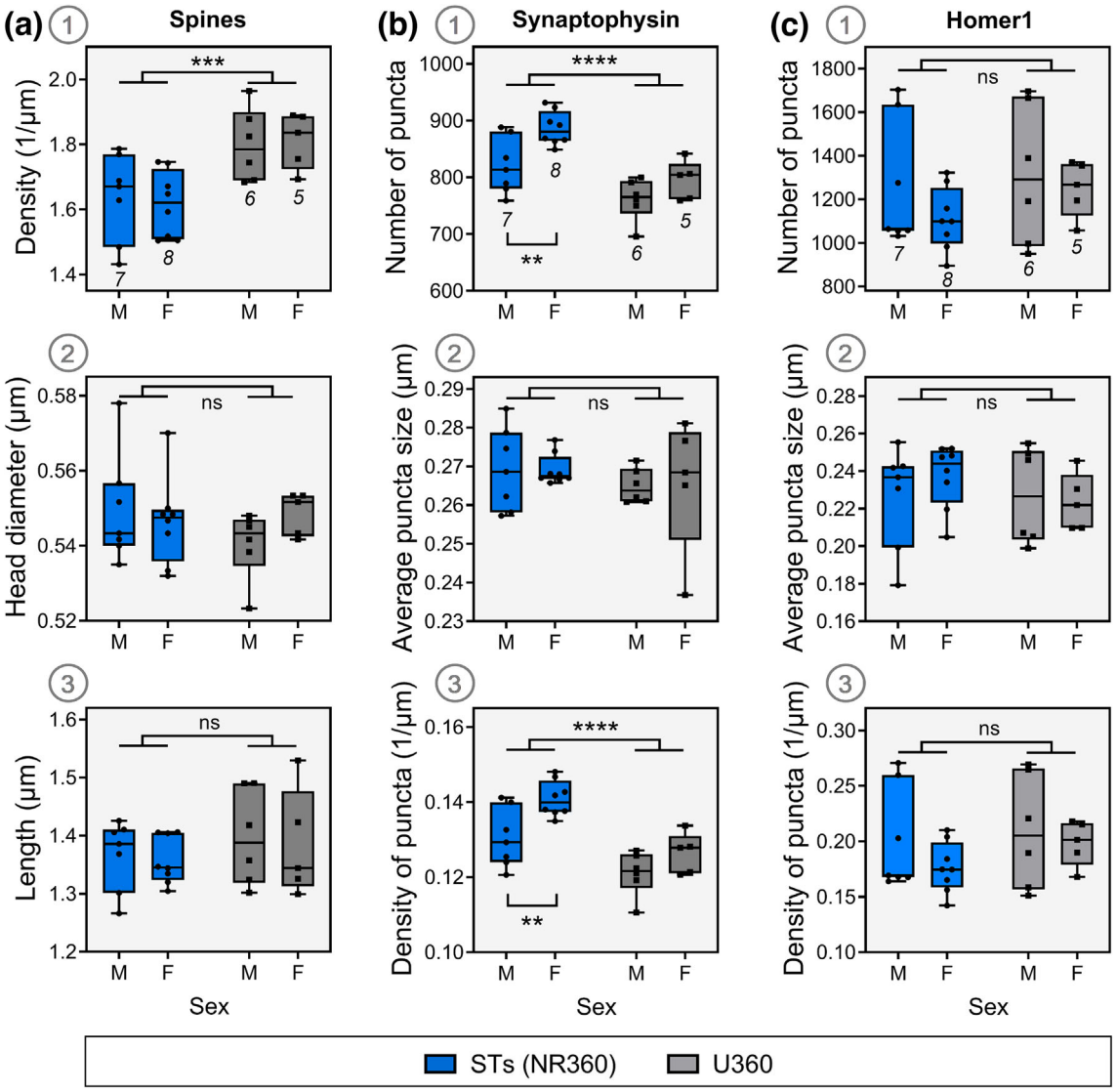

FIGURE 4 Effect of associative conditioning on dendritic spine measurements, pre- and post-synaptic activity. (a) Comparison of the density of spines [1], average spine head diameter [2] and average length of spines [3] between male and female STs of the NR group perfused 360 minutes after CS re-exposure (*blue circles*), and male and female unpaired rats (*grey rectangles*). (b) Comparison of synaptophysin staining between male and female STs of the NR group perfused 360 minutes after CS re-exposure, and male and female unpaired rats. (c) Comparison of homer1 staining between male and female STs of the NR group perfused 360 minutes after CS re-exposure, and male and female unpaired rats. (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

$r < 0.2908$, all $p > 0.2418$) on test day. No correlation was detected within the Unpaired group between synaptophysin staining and lever latency (Figure S7.A; all Pearson $r < 0.2181$, all $p > 0.5195$) or lever contacts (Figure S7.C; all Pearson $r < 0.2323$, all $p > 0.4919$) on test day.

Two-way ANOVAs comparing homer1 staining between STs from the NR360 group and Unpaired rats did not reveal any difference in the number, the size or the density of Homer1 puncta (Figure 4c; effect of training: all $F_{1,\ 22} < 1.045$, all $p > 0.3177$). For both NR360 STs and Unpaired rats, no major relationship was found between the latency to approach the CS (Figure S7.B; all Pearson or Spearman $r < -0.3870$, all $p > 0.0918$) or the number of contacts with CS (Figure S7.D; all Spearman $r < 0.2822$, all $p > 0.2280$) and homer1 staining.

When combining all conditioned animals of the NR group regardless of the sex or the time left before perfusion (Figure 5), rats who contacted the lever faster during the re-exposure session had a higher density of spines (Figure 5a-1; Pearson $r = -0.3596$, $p = 0.0165$) and longer spines (Figure 5a-3; Pearson $r = -0.3323$, $p = 0.0275$) in the NAc core. No correlation was found for spine head diameter (Figure 5a-2; Pearson $r = -0.0339$, $p = 0.8269$). No relationship was observed in Unpaired rats who did

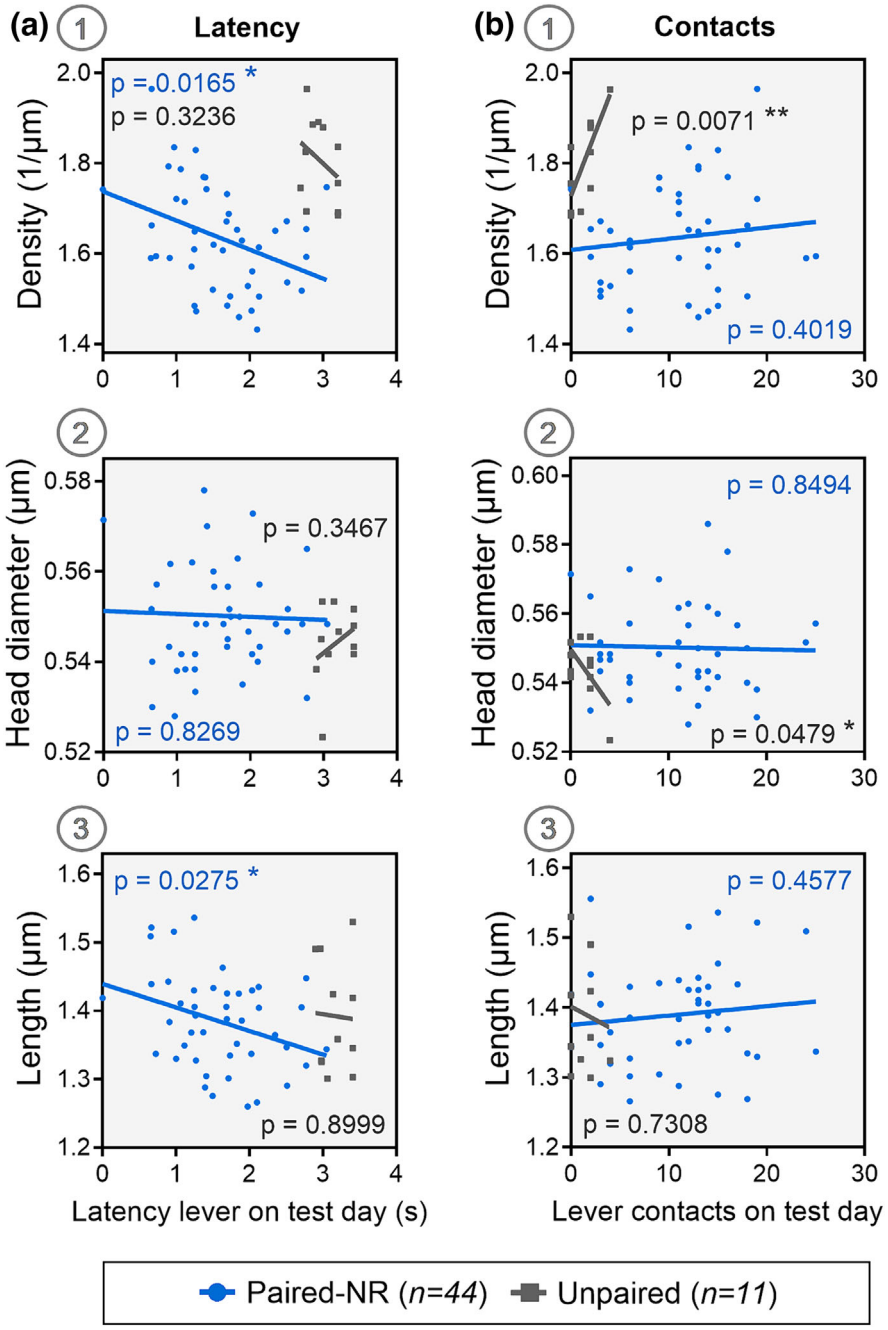

FIGURE 5 Relationship between spine measurements and individual lever-directed conditioned responses in paired and unpaired animals. (a) Correlation analysis between the latency to approach the CS during the non-rewarded cue re-exposure and the density [1], diameter [2] and length [3] of spines, in all paired-NR rats (*blue circles*) and all unpaired rats (*grey squares*). (b) Correlation analysis between the number of lever presses during the non-rewarded cue re-exposure and the density [1], diameter [2] and length [3] of spines, in all paired-NR rats and all unpaired rats. (* $p \leq 0.05$, ** $p \leq 0.01$).

not undertake conditioning between the rapidity to first contact with the lever and spine density (Figure 5a; Pearson $r = -0.3288$, $p = 0.3236$), diameter (Pearson $r = 0.3142$, $p = 0.3467$) and length (Pearson $r = -0.0431$, $p = 0.8999$). Figure 5b illustrates the relationship between the number of contacts with the lever during the re-exposure session and spine measurements in all animals of the NR group and in Unpaired rats. No correlation was found between Paired-NR rats who pressed the lever more and spine density (Figure 5b-1; Pearson $r = 0.1296$, $p = 0.4019$), spine head diameter (Figure 5b-2; Pearson $r = -0.02946$, $p = 0.8494$) or spine length (Figure 5b-3; Pearson $r = 0.01149$, $p = 0.4577$). No relationship was found between lever contacts and the length of spines in Unpaired animals (Figure 5b-3; Pearson $r = -0.1175$, $p = 0.7308$). However, Unpaired rats who pressed the lever more had a higher density of spines (Figure 5b-1; Pearson $r = 0.7557$, $p = 0.0071$) and a smaller spine head diameter (Figure 5b-2; Pearson $r = -0.6064$, $p = 0.0479$). This finding could suggest that the Unpaired rats have learned that the context has changed and no longer predicted reward (thus showing a similar effect to STs, as described above). On the other hand, no significant associations were observed between food cup approach and spine properties in Unpaired rats (Figure S6.C; all Spearman $r < 0.3272$, all $p > 0.3224$),

rats from the NR group (Figure S6.C; all Spearman $r < 0.1358$, all $p > 0.3794$) or animals from the *R* group (Figure S6.D; all Spearman $r < 0.1014$, all $p > 0.5226$).

3.3 | Impact of reward presence or absence during cue re-exposure on conditioned behaviour and neurobiology

Following the results of Experiment 1, Experiment 2 investigated whether the absence of reward during cue re-exposure might have impacted synaptic properties. Similar to Experiment 1, no difference was found in the neurobiology of animals perfused 30 minutes or 360 minutes after re-exposure to the CS (data not shown). Only the neurobiology of NR360 (Exp 1) and R360 (Exp 2) groups were therefore compared in the following section.

During the rewarded cue re-exposure, male and female STs interacted with the lever (Figure S3.A-1; effect of sex: $F_{1,\ 57} = 2.438$, $p = 0.1240$) and with the food cup (Figure S3.B-1-2; food cup entries: $F_{1,\ 57} = 2.869$, $p = 0.0958$; food cup latency: $F_{1,\ 57} = 1.547$, $p = 0.2187$) in a comparable manner. As in Experiment 1, no difference was found in the number of CS contacts (Figure S4.A; $\chi^2 = 5.162$, $df = 9$, $p = 0.8200$) made by STs of the R360 group between the beginning and the end of the re-exposure session. A significant difference was found for the latency to first contact the lever in R360 STs (Figure S4.A-2, *turquoise*; $\chi^2 = 18.72$, $df = 9$, $p = 0.0277$), however a post-hoc Dunn's multiple comparisons test specified that this was not due to a decrease of responses at the end of the re-exposure session, but was the result of the fourth trial diverging from other trials (trial 4 vs. 1: $p = 0.0151$; trial 4 vs. 9: $p = 0.0289$). When comparing the test session of Experiment 1 (NR), during which the cue was presented without the reward, and Experiment 2 (R), STs' number of lever presses (Figure S3.A-1; effect of reward: $F_{1,\ 57} = 0.1569$, $p = 0.6935$) and latency to contact the lever (Figure S3.A-2; effect of reward: $F_{1,\ 57} = 2.786$, $p = 0.1006$) were found to be equivalent. Sign-trackers for which the cue re-exposure session omitted the reward (NR) entered the food cup slower than rewarded animals (Figure S3.B-2; effect of reward: $F_{1,\ 57} = 7.735$, $p = 0.0073$). As neurobiological analyses focussed on STs, and because of the strong relationship detected between lever-directed responses (Figure S4.C; all Spearman $r < 0.7447$, $p \geq 0.0001$) and food cup-directed responses (Figure S6.B; Spearman $r = 0.6310$, $p < 0.0001$) displayed by the R360 group during the last training session and the cue re-exposure session, measures taken during the rewarded test session were considered to be reliable reflections of behavioural phenotypes.

A total of 191 proximal dendrites taken from random locations in the NAc core were reconstructed (5 to 6 per rat; average diameter = 0.97 μm; length ranging from 35 to 201.9 μm, average length = 85.78 μm). Consistent with Figure 4, when only considering animals perfused 360 minutes after the re-exposure session, a two-way ANOVA confirmed that Unpaired rats had a greater spine density than STs from the NR360 group (Figure 6a; effect of training: $F_{2,\ 32} = 6.868$, $p = 0.0033$; Šídák, $p = 0.0022$). A two-way ANOVA found a main effect of the reward on spine length (Figure 6a-3; $F_{2,\ 32} = 4.388$, $p = 0.0207$; Šídák, $p = 0.0161$) between STs who were only presented with the lever on the test session (NR360; Exp 1) and STs whose re-exposure session included the food reward (R360; Exp 2). No difference was found between both Paired groups in spine density (Šídák, $p = 0.3160$) or spine head diameter (effect of reward: $F_{2,\ 32} = 2.156$, $p = 0.1323$; Šídák, $p = 0.3444$). Unpaired rats did not significantly differ from rewarded R360 STs in their spine density (Šídák, $p = 0.1378$), spine diameter (effect of training: $F_{2,\ 32} = 2.156$, $p = 0.1323$) or spine length (Šídák, $p = 0.1611$). Male and female rats had comparable spine structural properties within and across training groups (effect of sex: all $F_{1,\ 32} < 0.1567$, all $p > 0.6948$).

Contrary to STs from the NR360 group which had more synaptophysin puncta (Figure 6b; effect of training: $F_{2,\ 33} = 5.825$, $p = 0.0068$; Šídák, $p = 0.0049$) and a greater density of synaptophysin puncta (effect of training: $F_{2,\ 33} = 5.824$, $p = 0.0068$; Šídák, $p = 0.0049$) than Unpaired rats, two-way ANOVAs revealed that animals from the R360 group were similar to Unpaired rats and to STs from the NR360 group in their amount of synaptophysin puncta (Figure 6b; Unpaired: Šídák, $p = 0.2483$; NR360: Šídák, $p = 0.1992$), average puncta size (effect of training: $F_{2,\ 33} = 0.5876$, $p = 0.5614$) and density of synaptophysin puncta (Unpaired: Šídák, $p = 0.2486$; NR360: Šídák, $p = 0.1990$). No significant sex difference was observed in R360 rats for the number of synaptophysin puncta ($F_{1,\ 33} = 0.5168$, $p = 0.4773$), the average puncta size ($F_{1,\ 33} = 0.08879$, $p = 0.7676$) or the density of synaptophysin puncta ($F_{1,\ 33} = 0.5168$; $p = 0.4773$). Although positive trends were observed between the latency to approach the CS and synaptophysin staining in R360 animals, no significant relationship was found (Figure S8.A; all Pearson $r < -0.4541$, $p > 0.0671$). A positive correlation was detected between the number of contacts made to the CS during the test session and the amount (Figure S8.C; Pearson $r = 0.5951$, $p = 0.0117$) and density (Pearson $r = 0.1820$, $p = 0.4846$) of synaptophysin puncta.

A two-way ANOVA showed that 360 minutes after the test session, the presentation of the reward (R360) did not lead to a change in homer1 number (Figure 6c; effect

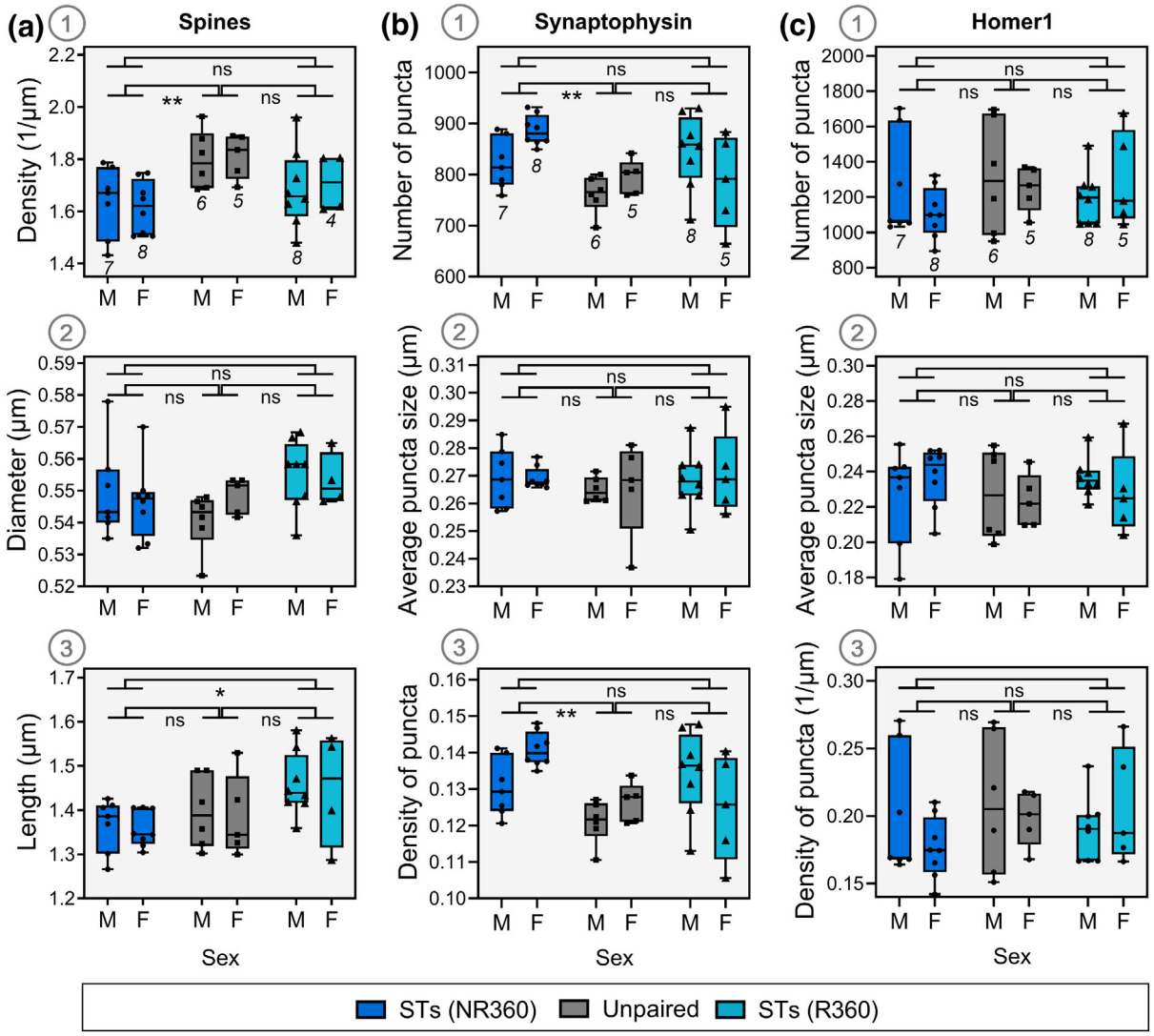

FIGURE 6 Effect of exposure to the reward during the test session on dendritic spine measurements, pre- and post-synaptic activity. (a) Comparison of the density [1] of spines, average spine head diameter [2] and average length of spines [3] between male and female STs of the NR group perfused 360 minutes after non-rewarded CS re-exposure (*blue circles*), male and female unpaired rats (*grey squares*), and male and female STs of the R group perfused 360 minutes after rewarded CS re-exposure (*turquoise triangles*). (b) Comparison of synaptophysin staining between male and female STs of the NR360 group, male and female unpaired rats, and male and female STs of the R360 group. (c) Comparison of homer1 staining between male and female STs of the NR360 group, male and female unpaired rats, and male and female STs of the R360 group. (* $p \leq 0.05$, ** $p \leq 0.01$).

of reward: $F_{2,\ 33} = 0.6130$, $p = 0.5478$), size (effect of reward: $F_{2,\ 33} = 0.4426$, $p = 0.6461$) or density of puncta (effect of reward: $F_{2,\ 33} = 0.6130$, $p = 0.5478$) in the NAc core. Homer1 staining was comparable between male and female R360 rats (effect of sex: all $F_{1,\ 33} < 0.2514$, all $p > 0.6194$). Similar to the NR group, no relationship was found between the latency to first approach the CS (Figure S8.B; all Pearson $r < 0.03955$, all $p > 0.8802$) or the number of CS contacts (Figure S8.D; all Pearson $r < 0.3732$, all $p > 0.1401$) and homer1 staining in R360 animals.

In contrast to Experiment 1, the speed at which Paired animals from the rewarded group (Paired-R) contacted the cue during the re-exposure was not associated with changes in spine density (Figure 7a; Spearman $r = -0.1367$, $p = 0.4481$), diameter (Spearman $r = -0.1538$, $p = 0.3927$) or length (Spearman $r = -0.2778$, $p = 0.1176$) 360 minutes after the session. The number of lever presses during the test session was not related to alterations in spine density (Figure 7b-1; Spearman $r = -0.137$, $p = 0.4481$), spine head diameter (Figure 7b-2; Spearman $r = -0.1538$, $p = 0.3927$) or

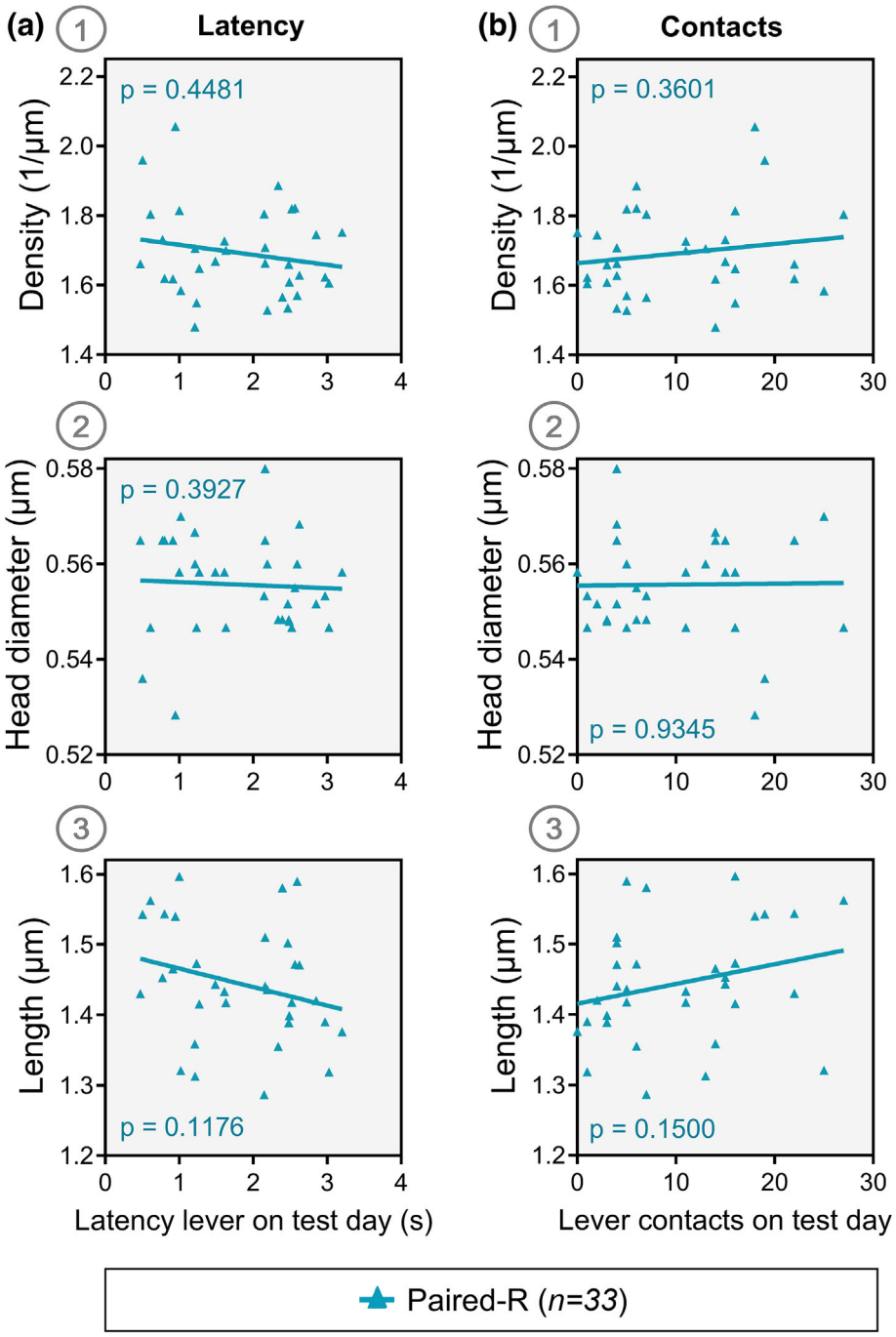

FIGURE 7 Relationship between spine measurements and individual lever-directed conditioned responses in animals re-exposed to the reward on test day. (a) Correlation analysis between the latency to approach the CS during the rewarded re-exposure and the density [1], diameter [2] and length [3] of spines, in all paired-R rats. (b) Correlation analysis between the latency to approach the CS during the rewarded re-exposure and the density [1], diameter [2] and length [3] of spines, in all paired-R rats.

spine length (Figure 7b-3; Spearman $r = -0.2778$, $p = 0.1176$).

3.4 | Relationship between oestrous cycle and incentive salience attribution to a Pavlovian cue

Results presented in this section combine animals from Experiment 1 (Paired-NR) and Experiment 2 (Paired-R) as they undertook identical training sessions. For this analysis, females from the 'proestrus' and 'not proestrus' groups are *not* the same for each session (Table S1); instead, each datapoint contains individuals that were or were not in proestrus on that specific day. Analyses of lever-directed and food cup-directed behaviours between males and females across the oestrous cycle are, therefore, simple two-way ANOVAs and not repeated measures.

CS-directed behaviour was similar between male STs, female STs in proestrus, and female STs in other stages through conditioning sessions (Figure 8a-1, 2, 3).

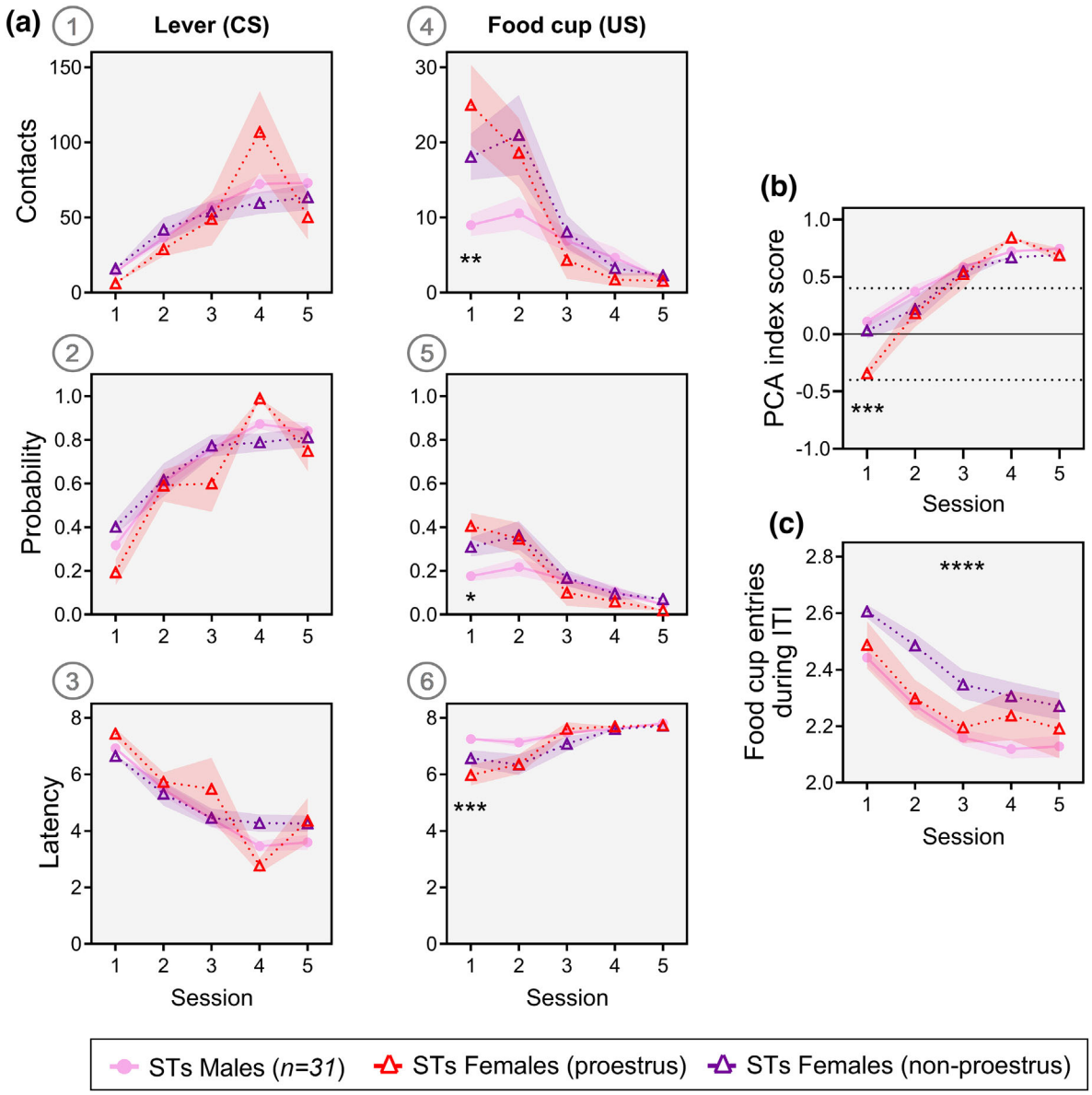

FIGURE 8 Conditioned responses across the oestrous cycle for paired animals. Datapoints from female groups are all composed of different individuals and are therefore not repeated measures. See Table S1 for the number of females in proestrus per session/datapoint. (a) Comparison of CS-directed behaviours [1, 2, 3] and US-directed behaviours [4, 5, 6] between female STs in proestrus, female STs in other oestrous stages, and male STs. All paired STs from the NR (non-rewarded) and R (rewarded) groups combined. (b) Evolution of the PCA index score of all male STs, of all female STs that were in proestrus during each specific session, and of all female STs that were in any other stages of the oestrous cycle during each specific session. (c) Total number of food cup entries during inter-trial intervals for each session in female STs in proestrus, female STs in other oestrous stages, and male STs. Data transformed using log10. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

However, differences transpired in food cup-directed behaviours (Figure 8a-4, 5, 6). The increased interaction with the food cup was previously observed at the beginning of training in female STs in Figure 3a appears to apply to all females regardless of their oestrous stage. Indeed, a two-way ANOVA showed that females in proestrus contacted the food cup during CS presentation significantly more than males (Figure 8a-4; effect of sex: $F_{2, 290} = 5.210$, $p = 0.0060$; Tukey, $p = 0.0008$), and they also contacted the food cup during CS presentation faster than males (Figure 8a-6; effect of sex: $F_{2, 246} = 8.055$, $p = 0.0004$; Tukey, $p = 0.0002$). Females in other oestrous stages visited the food magazine more (Figure 8a-4; Tukey: $p = 0.0048$) and faster (Figure 8a-6; Tukey: $p = 0.0.0173$) than males as well during CS presentation but, importantly, they did not differ from females in proestrus (food cup contacts: Tukey, $p = 0.2747$; food cup latency: Tukey, $p = 0.2121$). Congruent with previous

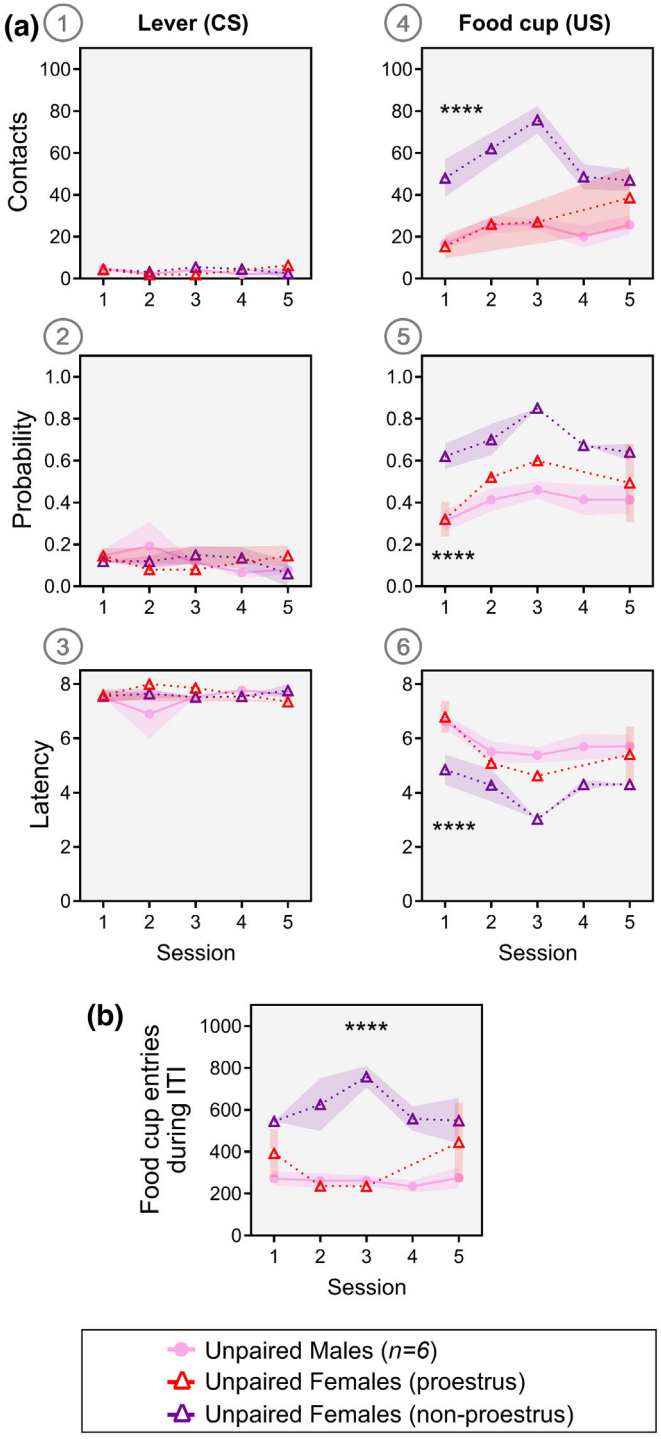

FIGURE 9 Responses across the oestrous cycle for unpaired animals. Datapoints from female groups are all composed of different individuals and are therefore not repeated measures. See Table S1 for the number of females in proestrus per session/datapoint. (a) Comparison of lever-directed behaviours [1, 2, 3] and food cup-directed behaviours [4, 5, 6] between unpaired females in proestrus, unpaired females in other oestrous stages, and unpaired males. (b) Number of food cup entries during inter-trial intervals in unpaired females in proestrus, unpaired females in other oestrous stages, and unpaired males. (**** $p < 0.0001$).

results, all STs displayed the same behaviour at the end of the Pavlovian procedure regardless of the oestrous cycle. Two-way ANOVAs found that female STs in proestrus were significantly different in their initial PCA index score (Figure 8b; effect of sex: $F_{2,\ 290} = 4.100$, $p = 0.0175$) that was calculated during the first conditioning session (Tukey: males vs. proestrus, $p = 0.0004$; proestrus vs. non-proestrus, $p = 0.0060$). Once the association between the CS and the US was learnt, male and all female STs scored similar PCA indices (Figure 8b). Figure 8c suggests that females that were not in proestrus appear to account for the difference observed in Figure 3b, wherein female STs visited the food cup during the inter-trial interval more than males during Pavlovian training (effect of sex: $F_{2,\ 290} = 27.22$, $p < 0.0001$; non-proestrus vs. males: Tukey, $p < 0.0001$; non-proestrus vs. proestrus: Tukey, $p = 0.0059$).

Unpaired control rats interacted with the lever at a comparable rate regardless of their sex and oestrous cycle (Figure 9a-1, 2, 3). A two-way ANOVA found that sex variations in food magazine-directed behaviour were previously observed in Figure 3a may be due to Unpaired females not in proestrus. Indeed, these females contacted the food cup during CS presentation more than males (Figure 9a-4; effect of sex: $F_{2,\ 48} = 40.85$, $p < 0.0001$; Tukey, $p < 0.0001$) and more than proestrus females (Tukey, $p < 0.0001$). Females in other oestrous stages also contacted the food cup during CS faster than males (Figure 9a-6; effect of sex: $F_{2,\ 48} = 18.05$, $p < 0.0001$; non-proestrus vs. males: Tukey, $p < 0.0001$) and faster than females in proestrus (Tukey, $p = 0.0029$). An effect of sex was detected in the probability of contact the food cup (Figure 9a-5; $F_{2,\ 48} = 25.11$, $p < 0.0001$) between Unpaired females not in proestrus and males (Tukey, $p < 0.0001$), and females in proestrus (Tukey, $p = 0.0017$). Similar to Paired animals, Unpaired females not in proestrus visited the food cup outside of lever presentation significantly more than females in proestrus (Figure 9b; effect of sex: $F_{2,\ 48} = 35.39$, $p < 0.0001$; Tukey, $p < 0.0001$) and more than males (Tukey, $p = 0.0005$).

4 | DISCUSSION

Characterising the neurobiological basis of conditioning offers a means to study the mechanisms by which reward-associated cues can, in some individuals, drive behaviour. The present work sought to investigate whether pre- and postsynaptic changes in the NAc core corresponded with variation in conditioned approach to a food-predictive cue in male and female rats, in the presence or absence of reward.

4.1 | Effect of associative conditioning and influence of reward on test day

Unexpectedly, in the absence of reward (Exp 1), on average STs – and particularly females – had greater synaptophysin expression, yet lower spine density, than Unpaired animals. Similar to previous research (Flagel et al., 2011; Yager et al., 2015), during our test day, rats experienced a new situation where the CS was presented for a shorter period (4 seconds rather than 8 seconds) and without reward in order to isolate the incentive properties of the cue from its predictive value. Incidentally, these differing stimulus durations might explain why a relationship between behavioural responses and spine properties was only detected for the latency to contact the CS and not for the number of CS contacts; a shorter lever extension time effectively put a ceiling on the total number of lever contacts possible.

To delve further into these results following the non-rewarded cue re-exposure, another cohort was exposed to both the lever and the food reward. Interestingly, the rewarded group (R360) showed longer spine length compared to the unrewarded group (NR360); this effect was analogous to the longer spines observed when collecting the brains at an earlier time point (NR30) compared to when animals were sacrificed six hours after the test session (NR360). Whilst additional comparisons are required (e.g., reward delivered to an Unpaired group during the final test), such findings may suggest that conditioning results in longer spines in the NAc, and that this effect is lost 360 minutes after re-exposure to the cue in the absence of reward.

The decrease in spine density (compared to Unpaired animals) and the decrease in spine length (compared to rewarded animals) in NR360 rats that unexpectedly did not receive a reward may also, in part, reflect new learning in the form of either a negative prediction error or extinction (Chang et al., 2016; Schultz et al., 1997). Although it requires further investigation, we did observe neuronal plasticity (upregulated synaptophysin), which could support such learning. Intriguingly, it is possible that synaptophysin was selectively upregulated within terminals that provide negative/inhibitory feedback onto spines in the NAc. Such regulation (e.g., cholinergic) might have blunted dopamine release at neuronal terminals projecting from the ventral tegmental area, thereby producing a local (NAc) cue-evoked reduction in dopamine and a negative prediction error (Melchior et al., 2015; Skirzewski et al., 2022; Zhou et al., 2001). Indeed, striatal dopamine and cholinergic transients may be anticorrelated, with a peak cholinergic response observed when cues are followed by the absence of reward (Chantranupong et al., 2023). Furthermore, the absence of reward could also lead to decreased glutamate signalling in the NAc (Guillory et al., 2022; Suto et al., 2013), which may have also impacted synaptic plasticity.

Arguments regarding prediction error explanations of the results may have limitations. For example, the R360 group received unexpected reward delivery 4 seconds after the presentation of the cue (instead of 8 seconds); this early reward might have resulted in a *positive* reward prediction error (Schultz et al., 1997). Accordingly, one might expect this positive prediction error to be associated with neuronal plasticity (although neurobiological changes associated with a slightly early reward are likely limited since the event has a different valence and may be less surprising compared to reward omission). Indeed, we show little evidence to support neurobiology underlying a positive prediction error (other than increased spine length, which, as noted above, is similar to the R30 findings). It should also be acknowledged that the neurobiology associated with a negative prediction error does not need to be paralleled by opposite neurobiology as a positive prediction error (e.g., dendritic spines may show different morphological changes, according to different timescales).

An alternative explanation for the lower level of plasticity observed in STs of the NR group is that extinction occurred due to the omission of the reward on test day. Sign-trackers are notoriously impervious to changes in the relationship between a pavlovian cue and its associated outcome and maintain cue-directed actions when the CS is no longer reinforced (Ahrens et al., 2016; Beckmann & Chow, 2015; Gillis & Morrison, 2019). In line with the literature, in the present study, we did not observe an extinction-related decrease in lever-directed behaviour throughout the test session and, overall, STs' behaviour towards the predictive cue remained similar in the presence or absence of reward during the test session. Yet, contrary to STs whose test session included the reward, STs for whom the reward was omitted (NR) interacted less with the food cup compared to the end of the Pavlovian training, and took a longer time to enter the food cup during the CS presentation. The most logical explanation for this is that NR rats did not enter the food cup on test day because there was no food to collect. However, it is possible that new learning might indeed have taken place; rats perhaps learnt that the lever no longer predicted reward, or that the reward was omitted because the sound of the food dispenser activating – an auditory cue – was missing.

4.2 | Neurobiology underlying variation in the strength of conditioning or incentive value

In the first experiment (which probed behaviour and neurobiology *without* reward), conditioned animals that

approached the lever faster during the re-exposure session possessed longer and denser dendritic spines in the NAc core. The positive correlation likely indicates that rats who more strongly associated the lever-cue with reward were also more resistant to the extinction conditions, or the change in the cue value, or could not process the negative prediction error. As mentioned previously, a spike in cholinergic activity is observed when a reward is omitted following cue re-exposure (Chantranupong et al., 2023). Importantly, STs have poorer control over striatal cholinergic signalling compared to GTs (Paolone et al., 2013). Therefore, it is possible that the most robust STs were not able to adapt their behaviour in the absence of reward, and that this could be the result of the inability of cholinergic signalling to modulate dendritic spine dynamics.

Alternatively, spinogenesis might explain increased spine density for Paired rats showing strong conditioned associations, and for unpaired animals who began contacting the lever during extinction (see discussion below). For either group, a new situation needs to be learnt (i.e., a context without reward) that could initiate the formation of new filopodia-like spines characterised by long necks and an absence of a head, believed to be precursors of excitatory spines (Hedrick et al., 2022; Yuste & Bonhoeffer, 2004). It is important to note that there were no positive correlations between the lever approach and spine characteristics for Experiment 2, during which the lever was presented *with* the reward. This lack of correlation likely reflects how substantial adaptations to behaviour were not needed (other than retrieving the food pellet earlier due to the shorter CS). Thus, the strength of sign-tracking may not reflect spine characteristics. Instead, individual variation in resistance to extinction (Experiment 1) may be associated with the synaptic properties we have observed.

A possible explanation for the lack of difference in homer1 puncta could reside in the fact that the postsynaptic density wherein homer1 proteins reside mostly follows the size of the spine (Arellano et al., 2007). Because no change in spine diameter was observed and newly formed spines do not possess a postsynaptic density, it is coherent that the latter, and thus homer1 puncta, would remain equivalent between STs and GTs. Alternatively, if presynaptic alterations did occur after re-exposure to the CS, proteins composing the postsynaptic density such as homer1 might not have had the time to change in response – although some molecular changes in presynaptic terminals and in the postsynaptic density are believed to occur in parallel (Sala et al., 2001).

As alluded above, for the Unpaired rats during the no-reward probe test, contacting the lever was positively correlated with spine density and negatively associated with spine head diameter. In combination, such results could suggest the growth of immature spines during this session when the lever was presented without reward for Unpaired animals. It is unclear what this means – and it might not be a reliable effect due to a relatively small sample size – but it could indicate that Unpaired rats learnt about the association between the operant chamber environment and the reward and that the session during which the lever was presented without reward could, similarly to NR STs, serve as contextual extinction for these animals. However, the food cup approach during the unrewarded cue re-exposure session was not associated with changes in dendritic spines for any rats; therefore, Unpaired rats may not have learnt that the context no longer predicted the reward.

4.3 | Sex differences and effect of the oestrous cycle on conditioned responding

Most investigations into sign- and goal-tracking that included both male and female individuals yielded mixed and inconsistent results (Dickson et al., 2015; Fuentes et al., 2018; Hughson et al., 2019; King et al., 2016; Kucinski et al., 2018; Madayag et al., 2017; Pitchers et al., 2015). Disparities between males and females in the speed or strength of conditioned responses in our experimental conditions were minor and mainly due to sample variations, thereby suggesting that the development of Pavlovian conditioned behaviours and the assignment of motivational value to discrete reward-associated cues is robust enough to neutralise potential innate sex differences. This is further supported by the uniformity of lever-directed and food cup-directed behaviours at the end of the Pavlovian training across the oestrous cycle, despite initial disparities.

Male and female rats mainly exhibited comparable neurobiological measures throughout the experiment, except for higher signs of presynaptic activity in female STs that were not re-exposed to the reward on test day. This could be related to elevated synaptophysin levels in females, and to sex differences in the processing of emotionally relevant information (Bangasser et al., 2011). In turn, greater synaptophysin levels in NR females might have facilitated the learning of the new situation encountered during the non-rewarded CS re-exposure, and slowed their approach behaviour.

The absence of variation in Pavlovian conditioned approach across the oestrous cycle has been described previously in the literature; however, the authors only compared the average coefficient of variance between males and females or the PCA score across the four oestrous stages (Madayag et al., 2017; Pitchers et al., 2015).

Because behaviours were similar once the association was learnt, it is tempting to hypothesise that the neurobiology of females might be the same regardless of their oestrous stage; however, the rate of learning was not comparable, and literature showed that spine plasticity mechanisms fluctuate throughout the cycle in the hippocampus and the NAc core (Warren et al., 1995; Woolley et al., 1990; Woolley & McEwen, 1994). This could be investigated by collecting the brains at different stages of the oestrous cycle after a PCA procedure.

The only consistent distinction between males and females in the present study was the number of food cup entries outside of lever presentation, which was higher in females regardless of whether animals learnt a Pavlovian association or not (i.e., in both conditioned and Unpaired female rats; the latter also entered the food cup at a higher rate *during* lever extension). This indicates that this sex difference may have been innate or related to food-reward learning, instead of cue-reward learning. Surprisingly, despite the fact that females are often described as being more active than males (Hyde & Jerussi, 1983; Tropp & Markus, 2001), most previous research found no such sex difference in magazine entries during inter-trial intervals (Fuentes et al., 2018; King et al., 2016; Pitchers et al., 2015 – but also see Hughson et al., 2019). The locomotion and the level of activity might thus not be the main elements involved in checking the food cup during trials in this experiment. Instead, animals might have simply exhibited a specific conditioned response driving them to stay near the food cup. Quantifying other patterns of behaviour besides the lever presses and the food cup entries (e.g., appetitive responses such as gnawing and biting, grooming and rearing) might allow us to widen the range of conditioned responses and to disentangle variation in non-specific behavioural activity.

4.4 | Degree of individual variation in conditioned responding

The ratio of STs, GTs and intermediate animals varies depending on the classification used by researchers (e.g., thresholds of the PCA index, or a percentage of animals with the most/least lever deflections: Fitzpatrick, Geary, et al., 2019; Flagel et al., 2007; Robinson & Flagel, 2009; Meyer et al., 2012; Yager & Robinson, 2013). The vendor, the strain/stock (Fitzpatrick et al., 2013; Pitchers et al., 2015) and early-life experience (Beckmann & Bardo, 2012; Fitzpatrick, Jagannathan, et al., 2019; Lomanowska et al., 2011), but also the length of the intertrial interval (Lee et al., 2018), have been shown to influence the development of these phenotypes. In Sprague–Dawley, the most widely used strain of rats, the proportion of STs typically ranges from 21% to 52%, and 14% to 39% of animals are usually categorised as GTs (Fitzpatrick, Geary, et al., 2019; Holden et al., 2024; Meyer et al., 2012; Meyer et al., 2014; Morrow et al., 2015; Saunders & Robinson, 2012; Singer, Bryan, et al., 2016; Yager & Robinson, 2013). However, a noticeably high number of STs was identified in the present study (55–77%: Figures S1 and S2). This might partly be due to us studying outbred Lister Hooded, which are not regularly assessed for variation in conditioned approach and are often described as particularly inquisitive and exploratory (Clemens et al., 2014). Alternatively, the housing experience of rats may be unique to our study; animals were received at three weeks old and developed together for six weeks. In contrast, in many other studies, there is a shorter acclimatisation period (Singer et al., 2009; Singer, Guptaroy, et al., 2016). In fact, current (unpublished) research from our laboratory exhibits relatively equal ST/GT/intermediate distributions when animals are tested approximately 1–2 weeks after arrival. Regardless, the present results suggest that long acclimatisation to housing conditions for Lister Hooded rats may be advantageous for future experiments focussing on sign-tracking behaviour.

4.5 | Limitations and caveats

Due to a low yield of GTs (who readily approach the food cup), we could not directly compare individuals for whom the predictive cue holds distinct values. Assessing dendritic spine plasticity and synaptic markers in GTs may have allowed us to clarify whether the changes we observed in STs are related to negative prediction errors, extinction, and perhaps more importantly, altered incentive value of the cue in the absence of reward. Such comparisons and conclusions would be facilitated by including additional control groups. For example, in the present study, Unpaired rats may have become conditioned to the training context (as indicated by a positive correlation between lever contacts and spine density on the no-reward test day), although similar effects were not observed when comparing food cup contacts. Thus, the Unpaired animals may not serve as a proper control group as presently trained and tested. Instead, future research may require control groups of rats that experience the operant chamber context repeatedly with or without reward delivery during training and who undergo a final test (before synaptic measurements) with or without reward delivery (at different time points). Other additional groups may include animals (e.g., Paired) that do not undergo any cue re-exposure

session before the perfusion. Whilst the results of such control experiments may help explain our novel findings, the design and utility of such studies must undergo ethical considerations, including how to reduce the number of animals used in research. Moreover, these control tests may have wide research implications beyond our laboratory, as other groups also study cue-evoked behaviour under reward omission (Flagel et al., 2011; Yager et al., 2015).

There may be other caveats or explanations for our findings which have yet to be discussed. Structural plasticity and synaptic mechanisms involved in processing the reward-associated cue were studied at two different time points after cue re-exposure (30 minutes vs. 360 minutes). There is a possibility that structural changes might have already occurred 30 minutes after the test session. Morphological alterations can sometimes be visible a few minutes after stimulation in both cell culture and in vivo (Engert & Bonhoeffer, 1999; Jourdain et al., 2003), although spinogenesis is generally noticeable approximately 30 minutes after long-term potentiation (LTP) induction (Abraham & Williams, 2003; Lamprecht & LeDoux, 2004) and in vivo spine growth is generally detectable hours after stimulation (De Roo et al., 2008). In vivo imaging could also enable measurements of how spines change across conditioning sessions. Such assessments may help determine if rats have unique cellular properties before training commences. Together, these reports suggest that earlier time points following cue exposure are worth investigating, as well as using additional methods for assessing synaptic plasticity.

4.6 | Conclusion

The present study supports the presence of disparities in the postsynaptic plasticity of MSNs in the NAc core. New questions are raised about how changes in spines may reflect variations in extinction learning and negative prediction errors. Perhaps most importantly, the studies will hopefully impact future work across behavioural neuroscience labs by emphasising the importance of environmental/contextual influences in controls, features of probe tests, sex differences, and the influence of the oestrous cycle on behaviour.

AUTHORS' CONTRIBUTION

MC: Data collection, formal analysis, draft of the manuscript. BFS, CLR, EJD, CJH: Conception and design of experiments, supervision, review and editing of the manuscript. IK: Design of experiments, experimental and technical assistance, review of the manuscript. AKS, IWB: Animal care, experimental and technical assistance.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to disclose.

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DATA AVAILABILITY STATEMENT

Data generated by these experiments can be accessed on the public access repository Figshare (https://doi.org/10.6084/m9.figshare.25396042).

ORCID

Morgane Colom https://orcid.org/0009-0000-6627-5283
Igor Kraev https://orcid.org/0000-0003-1822-278X
Agata K. Stramek https://orcid.org/0000-0003-0147-2707
Claire L. Rostron https://orcid.org/0000-0002-8863-9460
Christopher J. Heath https://orcid.org/0000-0003-3485-7128
Eleanor J. Dommett https://orcid.org/0000-0002-6973-8762
Bryan F. Singer https://orcid.org/0000-0003-3474-6137

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