EJN European Journal of Neuroscience FENS

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

Journal:	*European Journal of Neuroscience*
Manuscript ID	EJN-2023-07-30668.R1
Wiley - Manuscript type:	Research Report
Date Submitted by the Author:	n/a
Complete List of Authors:	Colom, Morgane; The Open University, School of Life, Health and Chemical Sciences; King's College London, Institute of Psychiatry, Psychology and Neuroscience; University of Cambridge, Department of Psychology Kraev, Igor; The Open University, School of Life, Health and Chemical Sciences Stramek, Agata; The Open University, School of Life, Health and Chemical Sciences Loza, Iwona; The Open University, School of Life, Health and Chemical Sciences Rostron, Claire; The Open University, School of Life, Health and Chemical Sciences Heath, Christopher; The Open University, School of Life, Health and Chemical Sciences Dommett, Eleanor; King's College London, Institute of Psychiatry, Psychology & Neuroscience Singer, Bryan; University of Sussex, School of Psychology
Special Section:	
Key Words:	synaptic plasticity, motivation, Pavlovian, sex differences, individual variation
Topic: Please choose the topic that most closely aligns with your manuscript. If your paper is an editorial, technical note or spotlight, select "other." (This is used for administrative purposes only.):	Behavioural Neuroscience

Title

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

Short running title

Synaptic plasticity of sign-tracker rats

Authors

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

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

[2]University of Sussex, School of Psychology, Sussex Neuroscience, Sussex Addiction Research and Intervention Centre, Brighton, United Kingdom.

[3]King's College, Institute of Psychiatry, Psychology and Neuroscience, London, United Kingdom.

*Present address: University of Cambridge, Department of Psychology, Cambridge, United Kingdom

Correspondence:

Dr Bryan F. Singer

E-mail: B.F.Singer@sussex.ac.uk

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

Word count: 8773

Figure count: 9

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.

Acknowledgements

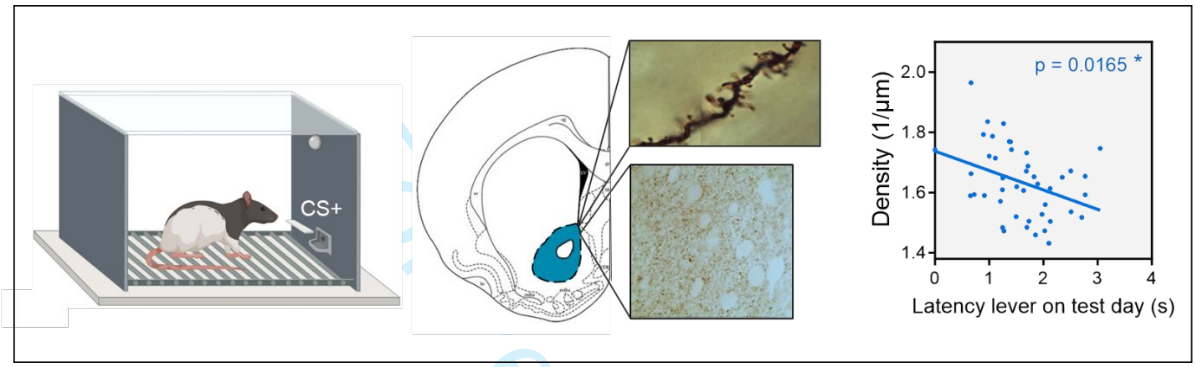
This work was funded by the Faculty of Science, Technology, Engineering and Mathematics (STEM) at The Open University. The authors would like to thank Karen Evans for technical assistance in the animal facility, and the University of Sussex for providing support with microscopy instrumentation.

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 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 nucleus accumbens core of male and female rats following a Pavlovian conditioned approach procedure. Data suggest that re-exposure to the food cue resulted in denser and longer spines in the NAc core of individuals that may have attributed more incentive salience to the cue. The presence of the reward during cue re-exposure also impacted spine morphology. In addition, male and female rats differed in behaviour and the expression of a marker of presynaptic activity (synaptophysin). Interestingly, the acquisition of conditioned responses in female sign-trackers was not affected by the oestrous cycle. This work supports the suggestion that individual variations in Pavlovian responses are associated with differences in neuronal plasticity of the nucleus accumbens core, whilst highlighting the importance of studying the behaviour and neurobiology of both male and female rats.

Graphical abstract

When attributed with motivational salience, reward-predictive stimuli can drive behaviour and contribute to maladaptive responses. We examined whether sign-tracking conditioned behaviours were associated with specific dendritic spine features, as well as markers of pre- and postsynaptic activity in the nucleus accumbens core of male and female rats. Data suggest that changes in accumbal neuronal plasticity may be related to individual variation in the responsiveness to Pavlovian cues.



Keywords

Dendritic spines; motivation; nucleus accumbens; rat; sign-tracking; synaptic plasticity.

Abbreviations

CR conditioned response; CS, conditioned stimulus; GT/GTs, goal-tracking/goal-trackers; ITI, inter-trial interval; 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.

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 and 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 and 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; Fig. 2.A), 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 *et al*., 2016a). The disruption of dopamine signalling in the NAc core specifically impairs the acquisition and the performance of conditioned responses in STs (Blaiss and Janak, 2009; Fraser and Janak, 2017; Saunders and Robinson, 2012), whilst stimulating dopaminergic neurotransmission preferentially enhances ST's approach towards the CS (Singer *et al.*, 2016b). 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 and 2016c). 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 *et al*., 2016c).

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 and Robinson, 2020; Casada *et al*., 1998; Morrow *et al*., 2011; Robinson *et al*., 2016; Saunders and 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 and 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 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; Petrovitch *et al.*, 2005).

Neurobiological and symptomatic sexual dimorphisms can be observed in conditions involving a dysfunctional salience of environmental cues (Becker and Chartoff, 2019; Fattore *et al.*, 2014; Kocane and 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.

Materials & Methods

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 12h-light, 12h-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; 331-513g for males, 197-369g 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).

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; Suppl. Video). 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).

Apparatus

Conditioning took place in 29.53 x 23.5 x 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.

Pavlovian conditioned approach

Pre-training. On day 1, Animals were familiarised with the food pellets used in the behavioural procedure (AIN-76A Rodent Tablet 45mg, TestDiet) by placing a handful in home cages two days before the beginning of training (Fig. 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 in a variable inter-trial interval (ITI) schedule (0-45 seconds range) after 5 minutes of acclimatisation in the dark. Rats were weighed before each daily session to monitor their welfare.

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 (Fig. 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 in a variable 90-seconds ITI schedule (30-150 seconds range).

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 (Fig. 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.

Cue re-exposure

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 (Fig. 1; Flagel *et al*., 2011; Yager *et al*., 2015). The lever was presented 10 times for 4 seconds in a variable 90-seconds ITI (30-150 seconds); no food pellets were delivered in the magazine following lever retraction.

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 in a variable 90-seconds ITI (30-150 seconds) and was immediately followed by the delivery of a food pellet (Fig. 1).

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 timepoints 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 (Fig. 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 (Fig. 1; de Roo, 2008). All Unpaired animals were perfused 360 minutes (6 hours) after lever re-exposure (Experiment 1, group **U360**; Fig. 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 0.1M phosphate-buffered saline (PBS; pH=7.4) and heparin, then with 3% paraformaldehyde (PFA) and 0.5% glutaraldehyde in 0.1M phosphate buffer (PB; pH=7.4).

Golgi-cox staining

Tissue preparation. Following fixation, coronal brain sections (100 µm) which included the NAc core were collected (Fig. 2.A; Leica VT1000 vibratome; RRID: SCR_016495) and

stored in 2.5% glutaraldehyde in 0.1M PB at +4°C until Golgi impregnation staining on single section (Gabbott and Somogyi, 1984). Three to six sections per animal were rinsed in 0.1M PB, processed in 1% osmium tetroxide and 0.2M 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 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.

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 (Fig. 2.A,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 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).

Synaptophysin and homer1 immunohistochemistry

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 0.1M, and kept at -20°C until immunostaining. Two to three sections per animal were left to warm at ambient temperature, then rinsed with 0.1M PB and left in 1% sodium borohydride for 30 minutes, after which the resulting effervescence was rinsed out with 0.1M PB. Peroxidase was blocked by placing the slices in 10% methanol and 3% hydrogen peroxide in 0.1M PB for 5 minutes. Sections were transferred in 5% skimmed milk in 0.1M PB for 15 minutes to block endogenous biotin, before being rinsed with 0.1M PB once more. Brain slices were then moved to a blocking buffer (0.5% Bovine Serum Albumin and 0.3% Triton X-100 in 0.1M 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# 101 011C3, RRID: AB_887822) for 36h on a gentle shaker at +4°C. Next, sections were rinsed in 0.1M 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 0.1M PB, and moved in ABC peroxidase (two drops of solution A and B for every 10 ml of 0.1% Bovine Serum Albumin in 0.1M PB) for 30 minutes. DAB in 30% hydrogen peroxide and 0.1M PB was used to develop staining and was subsequently rinsed with 0.1M 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.

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 (Fig. 2.C). Merged puncta smaller than 10 µm and larger than 200 µm were removed to exclude potential artefacts (Fig. 2.C). 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.

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 (Fig. 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).

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 α=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). 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 last test session between NR and R groups was 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 Suppl. Table 1) – 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 dendritic spine and synaptophysin analyses, respectively, due to damaged brain slices.

Results

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 (Fig. 1; Suppl. Fig. 1) and Experiment 2 (Fig. 1; Suppl. Fig. 2). When combining all STs from the NR (Exp 1) and R (Exp 2) Paired groups (Fig. 1; Fig. 3.A, *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 (Fig. 3.A-1; $F_{1, 59} = 0.5551$, $p = 0.4592$), lever probability (Fig. 3.A-2; $F_{1, 59} = 0.1720$, $p = 0.6799$) or lever latency (Fig. 3.A-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 (Fig. 3.A-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 (Fig. 3.A-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 (Fig. 3.A-5; $F_{4, 236} = 5.270$, $p = 0.0004$).

For the control Unpaired animals (Fig. 1), lever presentation and delivery of food pellets were not temporally associated. In accordance with the previous literature (Flagel *et al*., 2007 and 2011; Lomanovska *et al*., 2011; Meyer *et al*., 2012; Robinson and Flagel, 2009; Saunders *et al*., 2018; Singer *et al*., 2016a; Yager and Robinson, 2013), contrary to STs (Fig. 3.A) and GTs (data not shown), Unpaired rats neither learnt nor developed a conditioned response, as evidenced by the stable interaction with the lever and the food magazine across sessions (Fig. 3.A, *dotted lines*). Unpaired males and females exhibited equivalent lever-directed behaviours (Fig. 3.A). 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 (Fig. 3.A-4; $F_{1, 9} = 49.75$, $p < 0.0001$), latency (Fig. 3.A-6; $F_{1, 9} = 9.824$, $p = 0.0120$) and probability (Fig. 3.A-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 (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 3.B 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 found an effect of sex in STs ($F_{1, 59} = 17.82$, $p < 0.0001$). 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.

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; Fig. 1) undertook a single session during which they were re-exposed specifically to the CS (Fig. 1). 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). A Friedman test for repeated measures revealed no difference in lever contacts or latency to approach the lever across the 10 trials of the re-exposure session for NR360 animals, on which subsequent analyses will focus (Suppl. Fig. 3.A). A positive relationship was found in Paired animals between the last training session and the CS re-exposure (Suppl. Fig. 3.B). The correlation between 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 (Fig. 1; NR30) or 360 minutes (Fig. 1; NR360) following the re-exposure session, neurons from the NAc core (Fig. 2.A) 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 (Fig. 2.B). 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 (Fig. 2.C).

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 (Suppl. Fig. 4.A-3; $F_{1, 30} = 5.256$, $p = 0.0291$), rats did not greatly differ in their spine morphology (Suppl. Fig. 4.A), synaptophysin staining (Suppl. Fig. 4.B) or homer1 staining (Suppl. Fig. 4.C) 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 (Fig. 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 (Fig. 4.A-1; $F_{1, 22} = 16.12$, $p = 0.0006$). No difference was observed between both groups in spine length (Fig. 4.A-3; $F_{1, 22} = 1.103$, $p = 0.3050$) or average spine head diameter (Fig. 4.A-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 (Fig. 4.B). More specifically, STs had significantly more synaptophysin puncta (Fig. 4.B-1; effect of training: $F_{1, 22} = 24.21$, $p < 0.0001$) and a greater density of puncta (Fig. 4.B-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 (Fig. 4.B-1; effect of sex: $F_{1, 22} = 10.49$, $p = 0.0038$; Šídák, $p = 0.0065$) and had higher density (Fig. 4.B-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 (Suppl. Fig. 5.A) or the number of lever presses (Suppl. Fig. 5.C) 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 puncta (Fig. 4.C), and no major relationship was found between the latency to approach the CS (Suppl. Fig. 5.B) or the number of contacts with CS (Suppl. Fig. 5.D) and homer1 staining.

When combining all conditioned animals of the NR group regardless of the time left before perfusion and the sex (Fig. 5), rats who contacted the lever faster during the re-exposure session had a higher density of spines (Fig. 5.A-1; Pearson $r = -0.3596$, $p = 0.0165$) and longer spines (Fig. 5.A-3; Pearson $r = -0.3323$, $p = 0.0275$) in the NAc core. No correlation was found for spine head diameter (Fig. 5.A-2; Pearson $r = -0.0339$, $p = 0.8269$). No relationship was observed in Unpaired rats who did not undertake conditioning between the rapidity to first

contact the lever and spine density (Fig. 5.A; 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 5.B 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 (Fig. 5.B-1; Pearson r = 0.1296, p = 0.4019), spine head diameter (Fig. 5.B-2; Pearson r = -0.02946, p = 0.8494) or spine length (Fig. 5.B-3; Pearson r = 0.01149, p = 0.4577). Unpaired rats who pressed the lever more had a higher density of spines (Fig. 5.B-1; Pearson r = 0.7557, p = 0.0071) and a smaller spine head diameter (Fig. 5.B-2; Pearson r = -0.6064, p = 0.0479). No relationship was found between lever contacts and the length of spines in Unpaired animals (Fig. 5.B-3; Pearson r = -0.1175, p = 0.7308).

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 (Fig. 1) 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 NR360 (Fig. 1; Exp. 1) and R360 groups (Fig. 1; Exp. 2) were therefore compared in the following section.

As in Experiment 1, no difference was found in lever-directed behaviours made by sign-trackers of the R360 group between the beginning and the end of the re-exposure session (Suppl. Fig. 3.A). When comparing the test session of Experiment 1 (NR), during which the cue was presented without the reward, and Experiment 2 (R), STs' interaction towards the lever was found to be equivalent (Suppl. Fig. 3.C). The measures taken during the rewarded test session were considered to be reliable reflections of behavioural phenotypes due to the strong relationship detected between conditioned responses of the R360 group during the last training session and the cue re-exposure (Suppl. Fig. 3.D).

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). When only considering animals perfused 360 minutes after the re-exposure session, a two-way ANOVA found a main effect of the reward on spine length (Fig. 6.A-3; $F_{1, 24} = 9.772$, p = 0.0047) 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 groups in spine density (Fig. 6.A-1;

effect of reward: $F_{1, 23} = 1.945$, $p = 0.1794$) or spine head diameter (Fig. 6.A-2; effect of reward: $F_{1, 23} = 1.685$, $p = 0.2071$). Contrary to the NR360 group, spine properties did not statistically differ between STs of the R360 cohort and Unpaired animals (Suppl. Fig. 6.A).

Two-way ANOVAs comparing synaptophysin staining between STs of Experiment 1 (NR360) and 2 (R360) revealed reward x sex interactions between STs who were provided with the reward during re-exposure session. Indeed, female STs from the NR360 group had significantly more synaptophysin puncta (Fig. 6.B-1; interaction: $F_{1, 24} = 7.049$, $p = 0.0139$; Šídák, $p = 0.0169$) and a higher density of synaptophysin puncta (Fig. 6.B-3; interaction: $F_{1, 24} = 7.051$, $p = 0.0139$; Šídák, $p = 0.0169$) than female STs who were re-exposed to both the CS and the reward. No difference was found in males, and no main effect of reward was found between NR360 and R360 STs (Fig. 6.B; number of puncta: $F_{1, 24} = 2.534$, $p = 0.1245$; size of puncta: $F_{1, 24} = 0.01789$, $p = 0.8947$; density of puncta: $F_{1, 24} = 2.536$, $p = 0.1244$). Contrary to STs of the NR360 group (Fig. 4.B), female STs of the R360 group did not have more synaptophysin staining than their male counterparts (Fig. 6.B; effect of sex: $F_{1, 24} = 0.01409$, $p = 0.9065$). Contrary to Experiment 1, no difference in synaptophysin staining was observed between Unpaired rats and R360 animals (Suppl. Fig. 6.B). Although positive trends were observed between the latency to approach the CS and synaptophysin staining, no significant correlation was found (Suppl. Fig. 7.A). A positive correlation was detected between the size and density of synaptophysin puncta and the number of contacts made to the CS during the test session (Suppl. Fig. 7.C).

A two-way ANOVA showed that 360 minutes after the test session (Exp 2), the presentation of the reward did not lead to a change in homer1 number (Fig. 6.C-1; effect of reward: $F_{1, 24} = 0.5678$, $p = 0.4585$), size (Fig. 6.C-2; effect of reward: $F_{1, 24} = 0.02211$, $p = 0.8830$) or density of puncta (Fig. 6.C-3; effect of reward: $F_{1, 24} = 0.5678$, $p = 0.4585$) in the NAc core. Homer1 staining was comparable between Unpaired animals and STs from the R360 group (Suppl. Fig. 6.C). Similar to the NR group, no relationship was found between the latency to first approach the CS (Suppl. Fig. 7.B) or the number of CS contacts (Suppl. Fig. 7.D) and homer1 staining.

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 (Fig. 7.A; 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 (Fig. 7.B-1;

Spearman r = -0.137, p = 0.4481), spine head diameter (Fig. 7.B-2; Spearman r = -0.1538, p = 0.3927) or spine length (Fig. 7.B-3; Spearman r = -0.2778, p = 0.1176).

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

Results presented in this section combine animals from Experiment 1 (Paired-NR; Fig. 1) and Experiment 2 (Paired-R; Fig. 1) as they undertook identical training sessions. For this analysis, females from the 'proestrus' and 'not proestrus' groups are *not* the same for each session (Suppl Table 1); 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 (Fig. 8.A-1, 2, 3). However, differences transpired in food-cup directed behaviours (Fig. 8.A-4, 5, 6). The increased interaction with the food-cup previously observed at the beginning of training in female STs in Fig. 3.A 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 (Fig. 8.A-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 (Fig. 8.A-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 (Fig. 8.A-4; Tukey: p = 0.0048) and faster (Fig. 8.A-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 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 (Fig. 8.B; 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 (Fig. 8.B). Fig. 8.C suggests that females that were not in proestrus appear to account for the difference observed in Fig. 3.B, wherein female STs visited the food-cup during the ITI 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 (Fig. 1) interacted with the lever at a comparable rate regardless of their sex and oestrous cycle (Fig. 9.A-1, 2, 3). A two-way ANOVA found that sex variations in food magazine-directed behaviour previously observed in Fig. 3.A may be due to Unpaired females not in proestrus. Indeed, these females contacted the food-cup during CS presentation more than males (Fig. 9.A-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 (Fig. 9.A-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 to contact the food-cup (Fig. 9.A-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 (Fig. 9.B; effect of sex: $F_{2, 48} = 35.39$, $p < 0.0001$; Tukey, $p < 0.0001$) and more than males (Tukey, $p = 0.0005$).

Discussion

Characterising the neurobiological basis of sign-tracking 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 the motivation induced by a food-predictive cue in male and female rats.

Neurobiology underlying variation in incentive salience attribution

Animals that approached the lever faster during the re-exposure session, and that may therefore have attributed more incentive salience to the reward cue, possessed longer and denser dendritic spines in the NAc core. Spinogenesis might explain some aspects of this process. Indeed, when the need for more connections arises, the contact with pre-existing presynaptic terminals is typically initiated by newly formed 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 and Bonhoeffer, 2004). Newly formed spines must then stabilise into shorter mature spines, whose wider heads allow to support synaptic connections, in order for the network to become functional (Hotulainen and Hoogenraad, 2010).

Previous work demonstrated presynaptic signalling differences in STs compared to GTs (Flagel *et al*., 2011; Sarter and Phillips, 2018; Singer *et al.*, 2016a); we were unable to make this comparison due to a low number of GTs. Compared to STs, GTs may show less c-fos mRNA levels in the NAc, and regions that project to the NAc, after cue exposure (Flagel *et al*., 2011). Little is known about the relationship between c-Fos and synaptophysin, but it is possible that signalling cascades in activated neurons (*i.e*., c-Fos+) could promote synaptophysin expression. If this assumption is accurate, then it may be that GTs have lower synaptophysin expression after cue exposure. Regardless, our finding that ST rats with the greatest spine density and longer spines display the most robust lever approach supports the prediction that synaptic plasticity in the NAc core is positively associated with incentive motivation.

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).

Structural plasticity and synaptic mechanisms involved in processing the reward-associated cue were studied at two different timepoints after cue re-exposure (30 minutes vs. 360 minutes). Unexpectedly, only spine length appeared to have been altered in STs perfused 360 minutes after the non-rewarded re-exposure to the CS (Exp 1) compared to animals perfused earlier (Suppl. Fig. 4). This may be an indication of spine collapse (Halpain *et al.*, 1998; Zhou *et al.*, 2004), although it is difficult to explain this phenomenon without comparing to Unpaired animals similarly perfused 30 minutes after the re-exposure session. It is also worth noting that this difference was not found in the Paired-R group (Exp 2; data not shown). Spine density, spine head diameter, pre- and postsynaptic staining were similar between groups perfused 30 minutes and 360 minutes after the re-exposure session (Suppl. Fig. 4). 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 and Bonhoeffer, 1999; Jourdain *et al*., 2003), although spinogenesis is generally noticeable approximately 30 minutes after long-term potentiation (LTP) induction

(Abraham and Williams, 2003; Lamprecht and LeDoux, 2004) and *in vivo* spine growth is generally detectable hours after stimulation (De Roo, 2008).

Effect of associative conditioning and influence of reward on test day

Surprisingly, in the absence of reward (Exp 1), on average STs 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 (4s rather than 8s) 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. It is conceivable that changes in synaptic plasticity measured in Experiment 1 reflected new learning in the form of either a negative prediction error (*e.g*., an update of the association) or extinction, as both scenarios could impact dopaminergic activity (Chang *et al*., 2016; Schultz *et al*., 1997). Furthermore, spine parameters were found to be comparable between the Unpaired group and rewarded sign-trackers from the R360 group, as might be expected if the changes in plasticity rested on one of these two processes. The negative prediction error explanation may be preferred since STs are resistant to extinction (Ahrens *et al*., 2016; Beckman and Chow, 2015; Gillis *et al*., 2019). Indeed, we did not observe an extinction-related decrease in lever-directed behaviour, and STs behaved similarly in the presence or absence of reward during the test session.

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, markers of presynaptic activity appeared to be greater in females when the reward was omitted compared to when the reward was present. It is unclear why this is the case, but one possibility is that synaptophysin (which may be elevated in females; Bangasser *et al.*, 2011) was selectively upregulated within inhibitory terminals in the NAc. Increased inhibitory neurotransmitter levels (*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). Furthermore, the absence of reward could also lead to decreased glutamate signalling in the NAc (Guillory *et al.*, 2022; Suto *et al.*, 2013). These changes in neurotransmission may, in part, explain the decrease in spine density (compared to Unpaired animals) and the decrease in

spine length (compared to rewarded animals) in Paired rats that unexpectedly did not receive a reward; more research is needed on this topic. As mentioned above, a direct comparison between GTs and Unpaired animals might have allowed us to delve further into our surprising results. Assessing dendritic spine plasticity and synaptic markers in GTs would help us to understand whether the changes we observed in STs are related to negative prediction errors and, perhaps more importantly, altered incentive value of the cue in the absence of reward.

Related, and possibly a limitation for our interpretations (and those of other studies), is that Unpaired animals might not be an appropriate 'control' group. It is possible that rats are inherently STs or GTs, and conditioning is a tool to determine a rat's identity. In the present experiment, the vast majority of Paired animals were classified as STs; if these rats were *inherently* STs (*i.e.*, destined to become STs when trained), then one might assume that rats in the Unpaired groups were also likely *inherent* STs, but were not given the opportunity to express this behaviour (*i.e.*, they were not conditioned). Whilst challenging to test, our data might suggest that the *inherent* state of STs (as measured in Unpaired rats) could include elevated dendritic spine density. In this scenario, conditioning an *inherent* ST (*i.e.*, a Paired rat) might lead to a decrease in spine density in the NAc. This reduction could potentially be a compensatory mechanism responding to enhanced neurotransmission in the NAc, as suggested by synaptophysin levels in Paired rats and other studies measuring dopamine levels (Flagel *et al.*, 2011).

Another limitation of the current work is the absence of a control group that did not undergo any cue re-exposure session prior to the perfusion, which might have allowed to confirm or disprove the correlations between spine structural organisation and ST behaviour. Regardless, the inclusion of such additional Unpaired group rats may again run into the *inherent* ST problem discussed above, and, therefore, it may be unjustified and unethical to include additional subjects for this reason.

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 *et al.*, 2019a; Flagel *et al.*, 2007; Robinson and Flagel, 2009; Meyer *et al.*, 2012a; Yager and Robinson, 2013). The vendor, the strain/stock (Fitzpatrick *et al.*, 2013; Pitchers *et al.*, 2015) and early-life experience (Beckman and Bardo, 2012; Fitzpatrick *et al.*, 2019b; 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 *et al*., 2019a; Meyer *et al*., 2012a; Meyer *et al*., 2014; Morrow *et al*., 2015; Saunders and Robinson, 2012; Singer *et al*., 2016a; Yager and Robinson, 2013). However, a noticeably high number of STs was identified in the present study (55-77%: Suppl. Fig. 1, Suppl. Fig. 2). 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 and 2016a). 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.

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). Similarly, 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 (Suppl. Fig. 1 and 2), 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 also 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 sex differences in the processing of emotionally relevant information (Bangasser *et al.*, 2011).

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 and McEwen, 1994; Woolley *et al.*, 1990). 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 interaction with the food-cup 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 rats). 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 and Jerussi 1982; Tropp and Markus 2001), most previous research found no such sex difference in magazine entries during ITI (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.

Conclusion

The present study supports the presence of disparities in the postsynaptic plasticity of MSNs in the NAc core, reflecting individual variation in the tendency to attribute Pavlovian cues with incentive properties in male and female rats. Data suggest that re-exposure to the CS resulted in structural changes in dendritic spines in individuals with a tendency to sign-track. The timing of this plasticity needs to be further investigated to fully understand whether sign- and goal-trackers inherently differ, or whether the plasticity is altered as a result of Pavlovian training. One way to do this could be to pharmacologically inhibit dendritic spine structural plasticity through actin destabilisation (Toda *et al.*, 2010). Irrespective of future work, the current study provides insight into the neurobiological processes contributing to individual variation in the vulnerability to develop some neuropsychiatric disorders, particularly conditions involving anomalies in motivated behaviours and dopaminergic circuitry.

References

Abraham, W. C., & Williams, J. M. (2003). Properties and mechanisms of LTP maintenance. *The Neuroscientist, 9*(6)*, 463-474.*

Ahrens, A. M., Singer, B. F., Fitzpatrick, C. J., Morrow, J. D., & Robinson, T. E. (2016). Rats that sign-track are resistant to Pavlovian but not instrumental extinction. *Behavioural brain research*, *296, 418-430.*

Aitken, T. J., Greenfield, V. Y., & Wassum, K. M. (2016). Nucleus accumbens core dopamine signaling tracks the need-based motivational value of food-paired cues. *Journal of neurochemistry*, *136*(5)*, 1026-1036.*

Anselme, P., & Robinson, M. J. (2020). From sign-tracking to attentional bias: Implications for gambling and substance use disorders. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *99, 109861.*

Arellano, J. I., Benavides-Piccione, R., DeFelipe, J., & Yuste, R. (2007). Ultrastructure of dendritic spines: correlation between synaptic and spine morphologies. *Frontiers in neuroscience*, 10.

Bangasser, D. A., Zhang, X., Garachh, V., Hanhauser, E., & Valentino, R. J. (2011). Sexual dimorphism in locus coeruleus dendritic morphology: a structural basis for sex differences in emotional arousal. *Physiology & behavior*, *103*(3-4), 342-351.

Becker, J. B., Arnold, A. P., Berkley, K. J., Blaustein, J. D., Eckel, L. A., Hampson, E., Herman, J. P., Marts, S., Sadee, W., Steiner, M., Taylor, J., & Young, E. (2005). Strategies and methods for research on sex differences in brain and behavior. *Endocrinology, 146*(4)*, 1650-1673.*

Becker, J. B., & Chartoff, E. (2019). Sex differences in neural mechanisms mediating reward and addiction. *Neuropsychopharmacology*, *44*(1), 166-183.

Beckmann, J. S., & Bardo, M. T. (2012). Environmental enrichment reduces attribution of incentive salience to a food-associated stimulus. *Behavioural brain research, 226*(1), 331-334.

Beckmann, J. S., & Chow, J. J. (2015). Isolating the incentive salience of reward-associated stimuli: value, choice, and persistence. *Learning & memory, 22*(2), 116-127.

Berridge, K. C., & Robinson, T. E. (2003). Parsing reward. *Trends in neurosciences*, *26*(9), 507-513.

Blaiss, C. A., & Janak, P. H. (2009). The nucleus accumbens core and shell are critical for the expression, but not the consolidation, of Pavlovian conditioned approach. *Behavioural brain research*, *200*(1)*, 22-32.*

Cardinal, R. N., Parkinson, J. A., Hall, J., & Everitt, B. J. (2002). Emotion and motivation: the role of the amygdala, ventral striatum, and prefrontal cortex. *Neuroscience & Biobehavioral Reviews*, *26*(3)*, 321-352.*

Casada, J. H., Amdur, R., Larsen, R., & Liberzon, I. (1998). Psychophysiologic responsivity in posttraumatic stress disorder: generalized hyperresponsiveness versus trauma specificity. *Biological Psychiatry*, *44*(10), 1037-1044.

Chang, C. Y., Esber, G. R., Marrero-Garcia, Y., Yau, H. J., Bonci, A., & Schoenbaum, G. (2016). Brief optogenetic inhibition of dopamine neurons mimics endogenous negative reward prediction errors. *Nature neuroscience, 19*(1)*, 111-116.*

Clemens, L. E., Jansson, E. K. H., Portal, E., Riess, O., & Nguyen, H. P. (2014). A behavioral comparison of the common laboratory rat strains Lister Hooded, Lewis, Fischer 344 and Wistar in an automated homecage system. *Genes, Brain and Behavior, 13*(3)*, 305-321.*

Clifton, N. E., Trent, S., Thomas, K. L., & Hall, J. (2019). Regulation and function of activity-dependent homer in synaptic plasticity. *Complex Psychiatry*, *5*(3), 147-161.

De Roo, M., Klauser, P., & Muller, D. (2008). LTP promotes a selective long-term stabilization and clustering of dendritic spines. *PLoS biology, 6*(9)*, e219.*

Dickson, P. E., McNaughton, K. A., Hou, L., Anderson, L. C., Long, K. H., & Chesler, E. J. (2015). Sex and strain influence attribution of incentive salience to reward cues in mice. *Behavioural brain research, 292, 305-315.*

Engert, F., & Bonhoeffer, T. (1999). Dendritic spine changes associated with hippocampal long-term synaptic plasticity. *Nature, 399*(6731)*, 66-70.*

Fasano, C., Bourque, M. J., Lapointe, G., Leo, D., Thibault, D., Haber, M., Kortleven, C., DesGroseillers, L., Murai, K. K., & Trudeau, L. É. (2013). Dopamine facilitates dendritic spine formation by cultured striatal medium spiny neurons through both D1 and D2 dopamine receptors. *Neuropharmacology, 67, 432-443.*

Fattore, L., Melis, M., Fadda, P., & Fratta, W. (2014). Sex differences in addictive disorders. *Frontiers in neuroendocrinology*, *35*(3), 272-284.

Fitzpatrick, C. J., Geary, T., Creeden, J. F., & Morrow, J. D. (2019a). Sign-tracking behavior is difficult to extinguish and resistant to multiple cognitive enhancers. *Neurobiology of learning and memory*, *163*, 107045.

Fitzpatrick, C. J., Gopalakrishnan, S., Cogan, E. S., Yager, L. M., Meyer, P. J., Lovic, V., Saunders, B. T., Parker, C. C., Gonzales, N. M., Aryee, E., Flagel, S. B., Palmer, T. E., & Morrow, J. D. (2013). Variation in the form of Pavlovian conditioned approach behavior among outbred male Sprague-Dawley rats from different vendors and colonies: sign-tracking vs. goal-tracking. *PloS one, 8*(10)*, e75042.*

Fitzpatrick, C. J., Jagannathan, L., Lowenstein, E. D., Robinson, T. E., Becker, J. B., & Morrow, J. D. (2019b). Single prolonged stress decreases sign-tracking and cue-induced reinstatement of cocaine-seeking. *Behavioural brain research, 359*, 799-806.

Flagel, S. B., Akil, H., & Robinson, T. E. (2009). Individual differences in the attribution of incentive salience to reward-related cues: Implications for addiction. *Neuropharmacology, 56, 139-148.*

Flagel, S. B., Cameron, C. M., Pickup, K. N., Watson, S. J., Akil, H., & Robinson, T. E. (2011). A food predictive cue must be attributed with incentive salience for it to induce c-fos mRNA expression in cortico-striatal-thalamic brain regions. *Neuroscience, 196*, 80-96.

Flagel, S. B., Watson, S. J., Robinson, T. E., & Akil, H. (2007). Individual differences in the propensity to approach signals vs goals promote different adaptations in the dopamine system of rats. *Psychopharmacology, 191*(3)*, 599-607.*

Fraser, K. M., & Janak, P. H. (2017). Long-lasting contribution of dopamine in the nucleus accumbens core, but not dorsal lateral striatum, to sign-tracking. *European Journal of Neuroscience*, *46*(4)*, 2047-2055.*

Fuentes, S., Carrasco, J., Hatto, A., Navarro, J., Armario, A., Monsonet, M., Ortiz, J., & Nadal, R. (2018). Sex-dependent impact of early-life stress and adult immobilization in the attribution of incentive salience in rats. *PloS one, 13*(1)*, e0190044.*

Gabbott, P. L., & Somogyi, J. (1984). The 'single'section Golgi-impregnation procedure: methodological description. *Journal of neuroscience methods, 11*(4), 221-230.

Geinisman, Y., Berry, R. W., Disterhoft, J. F., Power, J. M., & Van der Zee, E. A. (2001). Associative learning elicits the formation of multiple-synapse boutons. *Journal of Neuroscience*, *21*(15)*, 5568-5573.*

Gillis, Z. S., & Morrison, S. E. (2019). Sign tracking and goal tracking are characterized by distinct patterns of nucleus accumbens activity. *ENeuro, 6*(2).

Gipson, C. D., Kupchik, Y. M., Shen, H., Reissner, K. J., Thomas, C. A., & Kalivas, P. W. (2013). Relapse induced by cues predicting cocaine depends on rapid, transient synaptic potentiation. *Neuron, 77*(5)*, 867-872.*

Grijalva, L. E., Miranda, M. I., & Paredes, R. G. (2021). Differential changes in GAP-43 or synaptophysin during appetitive and aversive taste memory formation. *Behavioural Brain Research*, *397*, 112937.

Guillory, A. M., Herrera, S. H., Baker, L. K., Bubula, N., Forneris, J., You, Z. B., Vezina, P., & Singer, B. F. (2022). Conditioned inhibition of amphetamine sensitization. *Neurobiology of Learning and Memory*, *192*, 107636.

Halpain, S., Hipolito, A., & Saffer, L. (1998). Regulation of F-actin stability in dendritic spines by glutamate receptors and calcineurin. *Journal of Neuroscience*, *18*(23), 9835-9844.

Hedrick, N. G., Lu, Z., Bushong, E., Singhi, S., Nguyen, P., Magaña, Y., Jilani, S., Lim, B. K., Ellisman, M., & Komiyama, T. (2022). Learning binds new inputs into functional synaptic clusters via spinogenesis. *Nature neuroscience*, *25*(6), 726-737.

Horner, A. E., Heath, C. J., Hvoslef-Eide, M., Kent, B. A., Kim, C. H., Nilsson, S. R., Oomen, C. A., Holmes, A., Saksida, L. M., & Bussey, T. J. (2013). The touchscreen operant platform for testing learning and memory in rats and mice. *Nature protocols*, *8*(10), 1961-1984.

Hotulainen, P., & Hoogenraad, C. C. (2010). Actin in dendritic spines: connecting dynamics to function. *Journal of Cell Biology*, *189*(4), 619-629.

Hughson, A. R., Horvath, A. P., Holl, K., Palmer, A. A., Solberg Woods, L. C., Robinson, T. E., & Flagel, S. B. (2019). Incentive salience attribution, "sensation-seeking" and "novelty-seeking" are independent traits in a large sample of male and female heterogeneous stock rats. *Scientific reports, 9*(1)*, 1-16.*

Hyde, J. F., & Jerussi, T. P. (1983). Sexual dimorphism in rats with respect to locomotor activity and circling behavior. *Pharmacology Biochemistry and Behavior, 18*(5)*, 725-729.*

Jourdain, P., Fukunaga, K., & Muller, D. (2003). Calcium/calmodulin-dependent protein kinase II contributes to activity-dependent filopodia growth and spine formation. *Journal of Neuroscience*, *23*(33)*, 10645-10649.*

Kalivas, P. W., & O'Brien, C. (2008). Drug addiction as a pathology of staged neuroplasticity. *Neuropsychopharmacology*, *33*(1), 166-180.

King, C. P., Palmer, A. A., Woods, L. C. S., Hawk, L. W., Richards, J. B., & Meyer, P. J. (2016). Premature responding is associated with approach to a food cue in male and female heterogeneous stock rats. *Psychopharmacology, 233*(13), 2593-2605.

Kokane, S. S., & Perrotti, L. I. (2020). Sex differences and the role of estradiol in mesolimbic reward circuits and vulnerability to cocaine and opiate addiction. *Frontiers in behavioral neuroscience*, *14*, 74.

Kucinski, A., Lustig, C., & Sarter, M. (2018). Addiction vulnerability trait impacts complex movement control: Evidence from sign-trackers. *Behavioural brain research, 350*, 139-148.

Lamprecht, R., & LeDoux, J. (2004). Structural plasticity and memory. *Nature Reviews Neuroscience*, *5*(1)*, 45.*

Leuner, B., Falduto, J., & Shors, T. J. (2003). Associative memory formation increases the observation of dendritic spines in the hippocampus. *Journal of Neuroscience, 23*(2), 659-665.

Lomanowska, A. M., Lovic, V., Rankine, M. J., Mooney, S. J., Robinson, T. E., & Kraemer, G. W. (2011). Inadequate early social experience increases the incentive salience of reward-related cues in adulthood. *Behavioural brain research*, *220*(1), 91-99.

Madayag, A. C., Stringfield, S. J., Reissner, K. J., Boettiger, C. A., & Robinson, D. L. (2017). Sex and adolescent ethanol exposure influence Pavlovian conditioned approach. *Alcoholism: Clinical and Experimental Research, 41*(4), 846-856.

Matsuzaki, M., Honkura, N., Ellis-Davies, G. C., & Kasai, H. (2004). Structural basis of long-term potentiation in single dendritic spines. *Nature, 429*(6993)*, 761-766.*

Melchior, J. R., Ferris, M. J., Stuber, G. D., Riddle, D. R., & Jones, S. R. (2015). Optogenetic versus electrical stimulation of dopamine terminals in the nucleus accumbens reveals local modulation of presynaptic release. *Journal of neurochemistry*, *134*(5), 833-844.

Meredith, G. E., Ypma, P., & Zahm, D. S. (1995). Effects of dopamine depletion on the morphology of medium spiny neurons in the shell and core of the rat nucleus accumbens. *Journal of Neuroscience*, *15*(5)*, 3808-3820.*

Meyer, P. J., Lovic, V., Saunders, B. T., Yager, L. M., Flagel, S. B., Morrow, J. D., & Robinson, T. E. (2012). Quantifying individual variation in the propensity to attribute incentive salience to reward cues. *PloS one, 7*(6)*, e38987.*

Morrow, J. D., Maren, S., & Robinson, T. E. (2011). Individual variation in the propensity to attribute incentive salience to an appetitive cue predicts the propensity to attribute motivational salience to an aversive cue. *Behavioural brain research, 220*(1)*, 238-243.*

Morrow, J. D., Saunders, B. T., Maren, S., & Robinson, T. E. (2015). Sign-tracking to an appetitive cue predicts incubation of conditioned fear in rats. *Behavioural brain research*, *276, 59-66.*

Ota, K. T., Monsey, M. S., Wu, M. S., & Schafe, G. E. (2010). Synaptic plasticity and NO-cGMP-PKG signaling regulate pre-and postsynaptic alterations at rat lateral amygdala synapses following fear conditioning. *PLoS One*, *5*(6), e11236.

Petrovich, G. D., Holland, P. C., & Gallagher, M. (2005). Amygdalar and prefrontal pathways to the lateral hypothalamus are activated by a learned cue that stimulates eating. *Journal of Neuroscience*, *25*(36), 8295-8302.

Pitchers, K. K., Flagel, S. B., O'Donnell, E. G., Woods, L. C. S., Sarter, M., & Robinson, T. E. (2015). Individual variation in the propensity to attribute incentive salience to a food cue: influence of sex. *Behavioural brain research, 278, 462-469.*

Pooley, A. E., Benjamin, R. C., Sreedhar, S., Eagle, A. L., Robison, A. J., Mazei-Robison, M. S., Breedlove, S. M., & Jordan, C. L. (2018). Sex differences in the traumatic stress response: PTSD symptoms in women recapitulated in female rats. *Biology of sex differences*, *9*, 1-11.

Robinson, M. J. F., Fischer, A. M., Ahuja, A., Lesser, E. N., & Maniates, H. (2016). Roles of "wanting" and "liking" in motivating behavior: gambling, food, and drug addictions. *Behavioral neuroscience of motivation*, 105-136.

Robinson, T. E., & Flagel, S. B. (2009). Dissociating the predictive and incentive motivational properties of reward-related cues through the study of individual differences. *Biological psychiatry*, *65*(10), 869-873.

Sala, C., Piëch, V., Wilson, N. R., Passafaro, M., Liu, G., & Sheng, M. (2001). Regulation of dendritic spine morphology and synaptic function by Shank and Homer. *Neuron*, *31*(1), 115-130.

Sarter, M., & Phillips, K. B. (2018). The neuroscience of cognitive-motivational styles: Sign-and goal-trackers as animal models. *Behavioral Neuroscience, 132*(1)*, 1.*

Saunders, B. T., & Robinson, T. E. (2010). A cocaine cue acts as an incentive stimulus in some but not others: implications for addiction. *Biological psychiatry, 67*(8)*, 730-736.*

Saunders, B. T., & Robinson, T. E. (2012). The role of dopamine in the accumbens core in the expression of Pavlovian-conditioned responses. *European Journal of Neuroscience*, *36*(4)*, 2521-2532.*

Saunders, B. T., Richard, J. M., Margolis, E. B., & Janak, P. H. (2018). Dopamine neurons create Pavlovian conditioned stimuli with circuit-defined motivational properties. *Nature neuroscience*, *21*(8)*, 1072-1083.*

Schultz, W., Dayan, P., & Montague, P. R. (1997). A neural substrate of prediction and reward. *Science, 275*(5306)*, 1593-1599.*

Singer, B. F., Bryan, M. A., Popov, P., Scarff, R., Carter, C., Wright, E., Aragona B. J., & Robinson, T. E. (2016a). The sensory features of a food cue influence its ability to act as an incentive stimulus and evoke dopamine release in the nucleus accumbens core. *Learning & Memory*, *23*(11)*, 595-606.*

Singer, B. F., Bubula, N., Li, D., Przybycien-Szymanska, M. M., Bindokas, V. P., & Vezina, P. (2016c). Drug-paired contextual stimuli increase dendritic spine dynamics in select nucleus accumbens neurons. *Neuropsychopharmacology, 41*(8), 2178-2187.

Singer, B. F., Guptaroy, B., Austin, C. J., Wohl, I., Lovic, V., Seiler, J. L., Vaughan, R. A., Gnegy, M. R., Robinson, T. E., & Aragona, B. J. (2016b). Individual variation in incentive salience attribution and accumbens dopamine transporter expression and function. *European Journal of Neuroscience*, *43*(5)*, 662-670.*

Singer, B. F., Tanabe, L. M., Gorny, G., Jake-Matthews, C., Li, Y., Kolb, B., & Vezina, P. (2009). Amphetamine-induced changes in dendritic morphology in rat forebrain correspond to associative drug conditioning rather than nonassociative drug sensitization. *Biological psychiatry, 65*(10), 835-840.

Skirzewski, M., Princz-Lebel, O., German-Castelan, L., Crooks, A. M., Kim, G. K., Tarnow, S. H., Reichelt, A., Memar, S., Palmer, D., Li, Y., Rylett, R. J., Saksida, L. M., Prado, V. F., Prado, M. A. M., & Bussey, T. J. (2022). Continuous cholinergic-dopaminergic

updating in the nucleus accumbens underlies approaches to reward-predicting cues. *Nature Communications*, *13*(1), 7924.

Stramek, A. K., Johnson, M. L., & Taylor, V. J. (2019). Improved timed-mating, non-invasive method using fewer unproven female rats with pregnancy validation via early body mass increases. *Laboratory animals, 53*(2)*, 148-159.*

Suto, N., Elmer, G. I., Wang, B., You, Z. B., & Wise, R. A. (2013). Bidirectional modulation of cocaine expectancy by phasic glutamate fluctuations in the nucleus accumbens. *Journal of Neuroscience*, *33*(21), 9050-9055.

Toda, S., Shen, H., & Kalivas, P. W. (2010). Inhibition of actin polymerization prevents cocaine-induced changes in spine morphology in the nucleus accumbens. *Neurotoxicity research*, *18*, 410-415.

Tropp, J., & Markus, E. J. (2001). Sex differences in the dynamics of cue utilization and exploratory behavior. *Behavioural brain research, 119*(2)*, 143-154.*

Warren, S. G., Humphreys, A. G., Juraska, J. M., & Greenough, W. T. (1995). LTP varies across the estrous cycle: enhanced synaptic plasticity in proestrus rats. *Brain research*, *703*(1-2), 26-30.

Woolley, C. S., & McEwen, B. S. (1994). Estradiol regulates hippocampal dendritic spine density via an N-methyl-D-aspartate receptor-dependent mechanism. *Journal of Neuroscience*, *14*(12)*, 7680-7687.*

Woolley, C. S., Gould, E., Frankfurt, M., & McEwen, B. S. (1990). Naturally occurring fluctuation in dendritic spine density on adult hippocampal pyramidal neurons. *Journal of neuroscience*, *10*(12)*, 4035-4039.*

Yager, L. M., & Robinson, T. E. (2013). A classically conditioned cocaine cue acquires greater control over motivated behavior in rats prone to attribute incentive salience to a food cue. *Psychopharmacology, 226*(2)*, 217-228.*

Yager, L. M., Pitchers, K. K., Flagel, S. B., & Robinson, T. E. (2015). Individual variation in the motivational and neurobiological effects of an opioid cue. *Neuropsychopharmacology*, *40*(5)*, 1269-1277.*

Yao, W. D., Spealman, R. D., & Zhang, J. (2008). Dopaminergic signaling in dendritic spines. *Biochemical pharmacology, 75*(11)*, 2055-2069.*

Yuste, R., & Bonhoeffer, T. (2004). Genesis of dendritic spines: insights from ultrastructural and imaging studies. *Nature Reviews Neuroscience*, *5*(1), 24-34.

Zhou, F. M., Liang, Y., & Dani, J. A. (2001). Endogenous nicotinic cholinergic activity regulates dopamine release in the striatum. *Nature neuroscience*, *4*(12), 1224-1229.

Zhou, Q., Homma, K. J., & Poo, M. M. (2004). Shrinkage of dendritic spines associated with long-term depression of hippocampal synapses. *Neuron*, *44*(5), 749-757.

Figure legends

Fig. 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 timepoints 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.

Fig. 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.

Fig. 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: # $p \leq 0.05$, ## $p \leq 0.01$; #### $p < 0.0001$).

Fig. 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. (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

Fig. 5. Relationship between spine measurements and individual 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. (* $p \leq 0.05$, ** $p \leq 0.01$).

Fig. 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*), 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, and male and female STs of the R360 group. (C) Comparison of homer1 staining between male and female STs of the NR360 group, and male and female STs of the R360 group. (* $p \leq 0.05$, ** $p \leq 0.01$).

Fig. 7. Relationship between spine measurements and individual 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.

Fig. 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 Suppl. Table 1 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. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

Fig. 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 Suppl. Table 1 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. (**** $p < 0.0001$).

Supporting information

Additional supporting information may be found in the Web version of this article:

Suppl. Fig. 1. Pavlovian phenotypic repartition of Experiment 1 and evolution across training.

Suppl. Fig. 2. Pavlovian phenotypic repartition of Experiment 2 and evolution across training.

Suppl. Fig. 3. Rewarded and non-rewarded CS re-exposure.

Suppl. Fig. 4. Effect of the time of perfusion on dendritic spine measurements, pre- and postsynaptic activity within the NR group.

Suppl. Fig. 5. Relationship between pre- and postsynaptic activity and conditioned responses in Paired-NR and Unpaired animals.

Suppl. Fig. 6. Effect of exposure to the reward during the test session on dendritic spine measurements, pre- and post-synaptic activity.

Suppl. Fig. 7. Relationship between pre- and postsynaptic activity and conditioned responses in rewarded and unrewarded Paired animals.

Suppl. Table 1. Quantification of the oestrous cycle repartition in females of Experiments 1 and 2.

Suppl. Video 1. Illustration of the 'oestrous dance' exhibited by female rats in proestrus.

Conflict of interest statement

The authors have no conflicts of interest to disclose.

Data availability statement

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

Figure legends

Fig. 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 timepoints 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.

Fig. 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.

Fig. 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: # $p \leq 0.05$, ## $p \leq 0.01$; #### $p < 0.0001$).

Fig. 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. (** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

Fig. 5. Relationship between spine measurements and individual 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. (* $p \leq 0.05$, ** $p \leq 0.01$).

Fig. 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*), 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, and male and female STs of the R360 group. (C) Comparison of homer1 staining between male and female STs of the NR360 group, and male and female STs of the R360 group. (* $p \leq 0.05$, ** $p \leq 0.01$).

Fig. 7. Relationship between spine measurements and individual 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.

Fig. 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 Suppl. Table 1 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. (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p < 0.0001$).

Fig. 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 Suppl. Table 1 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. (**** $p < 0.0001$).

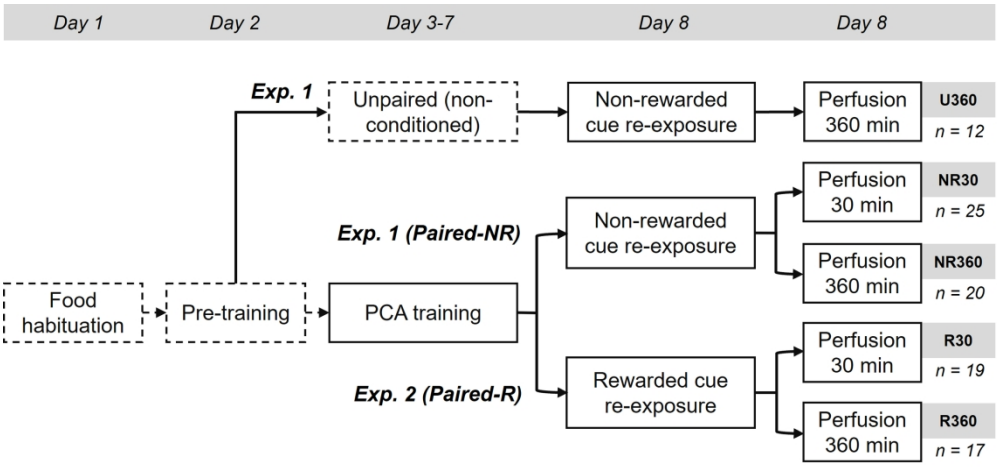


Fig. 1. Schematic illustration of the experimental timeline.

200x93mm (300 x 300 DPI)

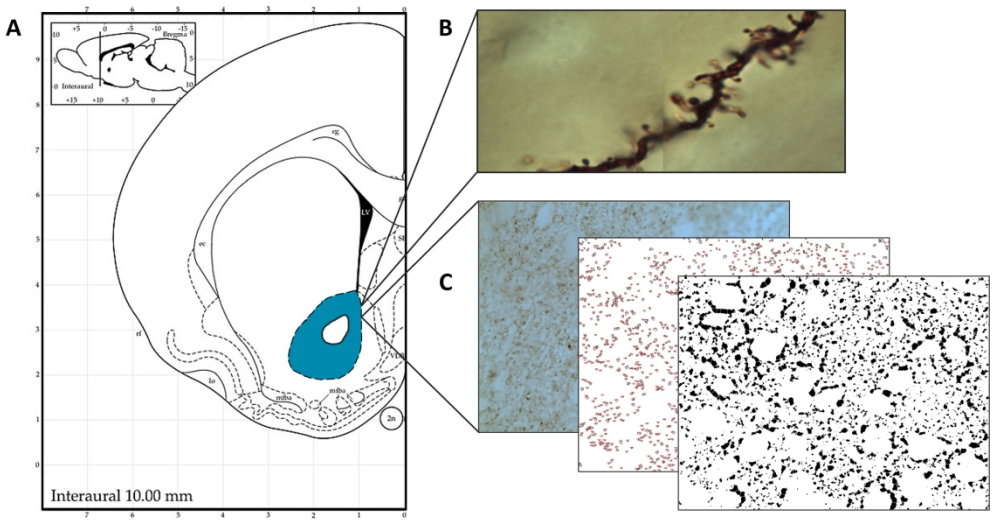


Fig. 2. Dendritic spines and presynaptic markers in the NAc core.

247x131mm (300 x 300 DPI)

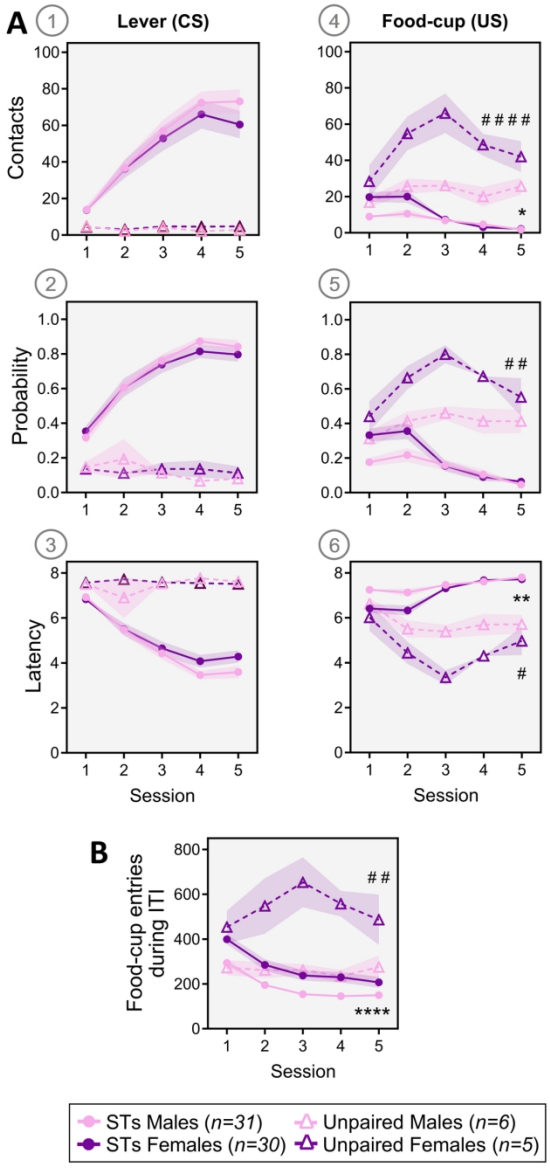


Fig. 3. Interaction with the CS+ and the US during training.

54x101mm (600 x 600 DPI)

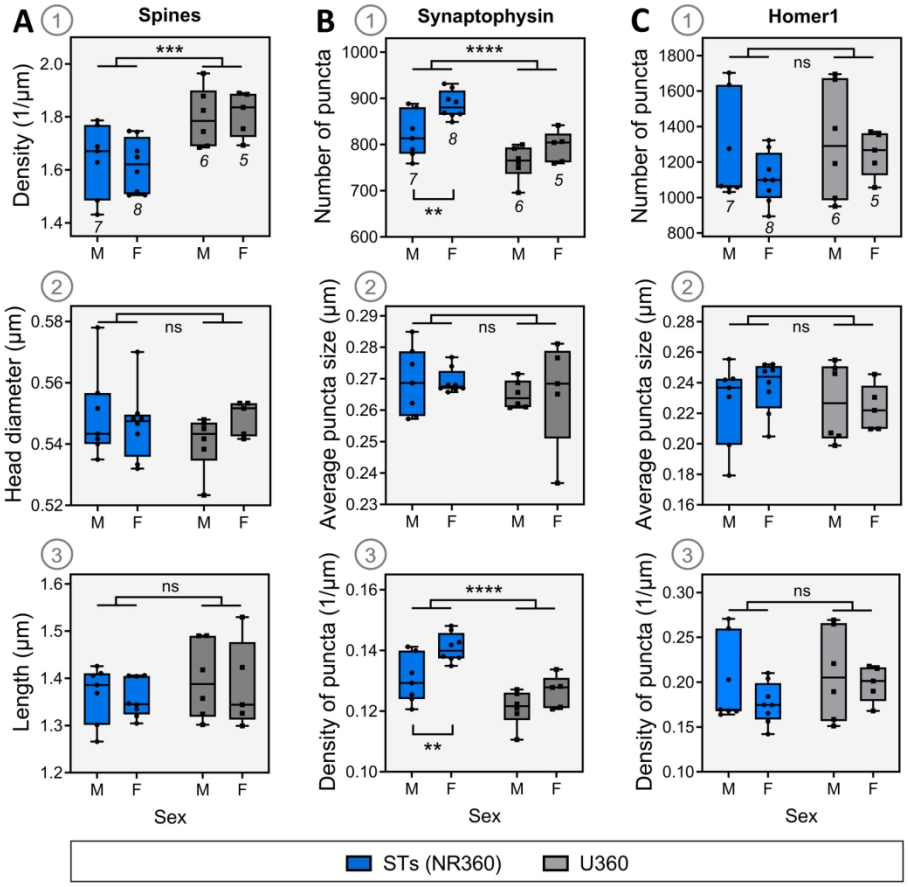


Fig. 4. Effect of associative conditioning on dendritic spine measurements, pre- and post-synaptic activity.

82x75mm (600 x 600 DPI)

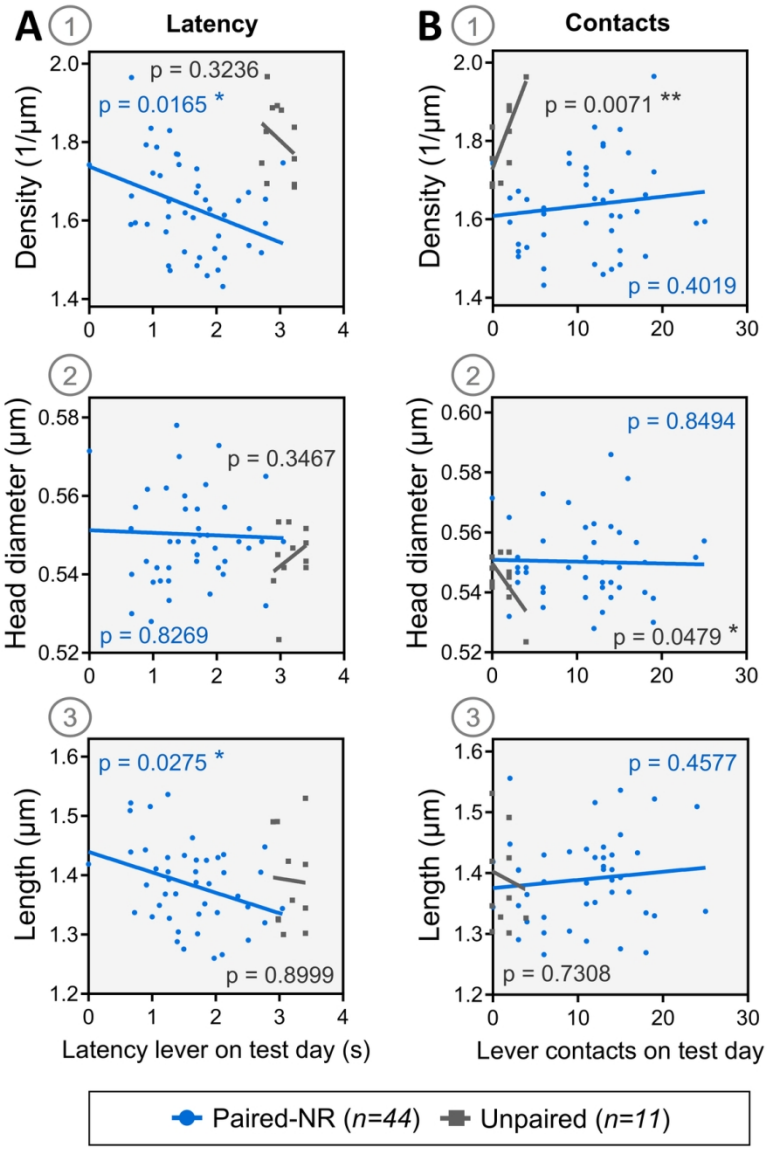


Fig. 5. Relationship between spine measurements and individual conditioned responses in Paired and Unpaired animals.

56x76mm (600 x 600 DPI)

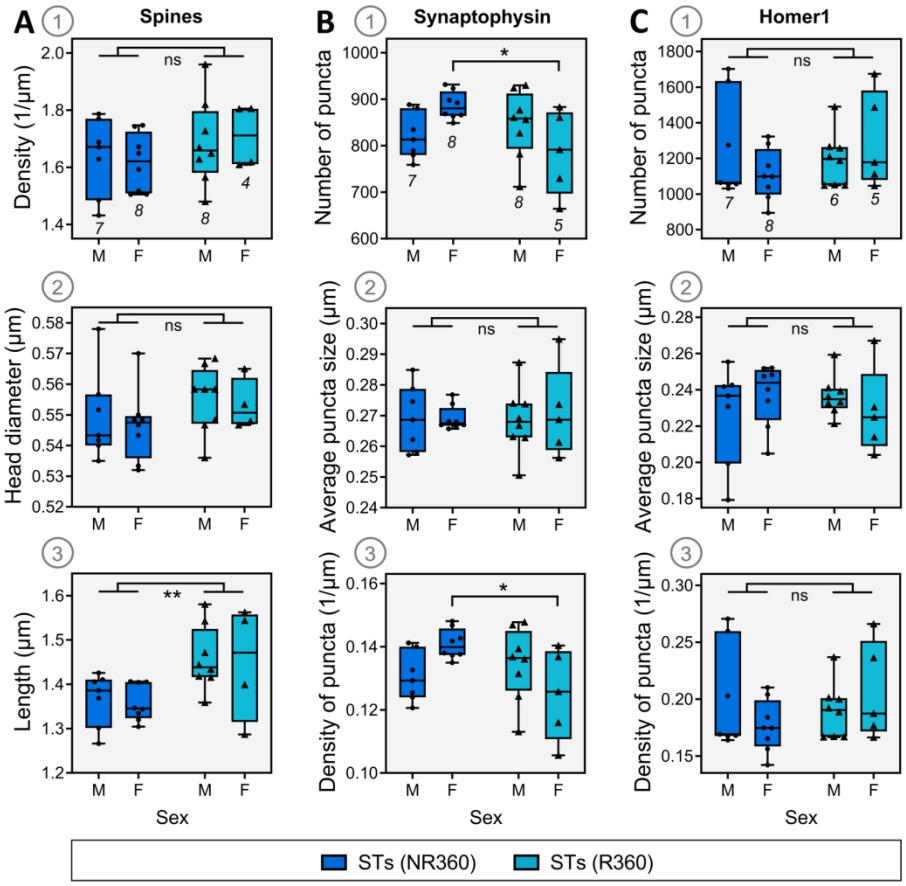


Fig. 6. Effect of exposure to the reward during the test session on dendritic spine measurements, pre- and post-synaptic activity.

82x75mm (600 x 600 DPI)

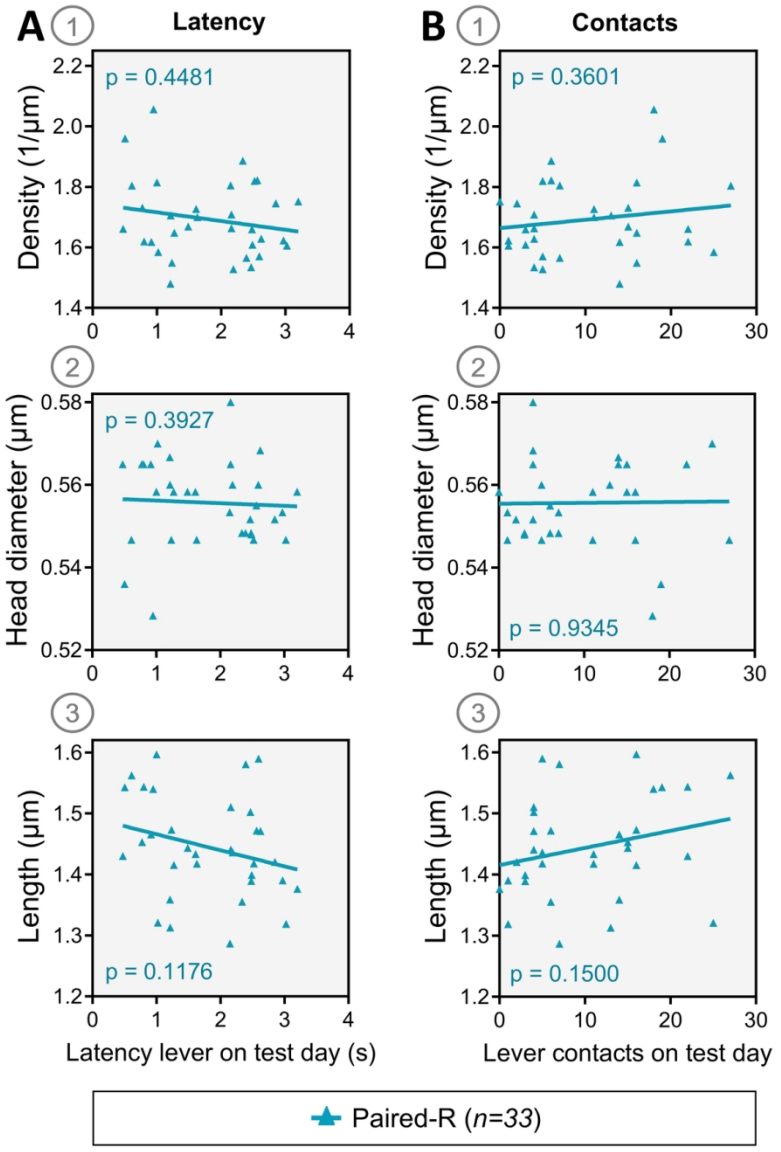


Fig. 7. Relationship between spine measurements and individual conditioned responses in animals re-exposed to the reward on test day.

56x76mm (600 x 600 DPI)

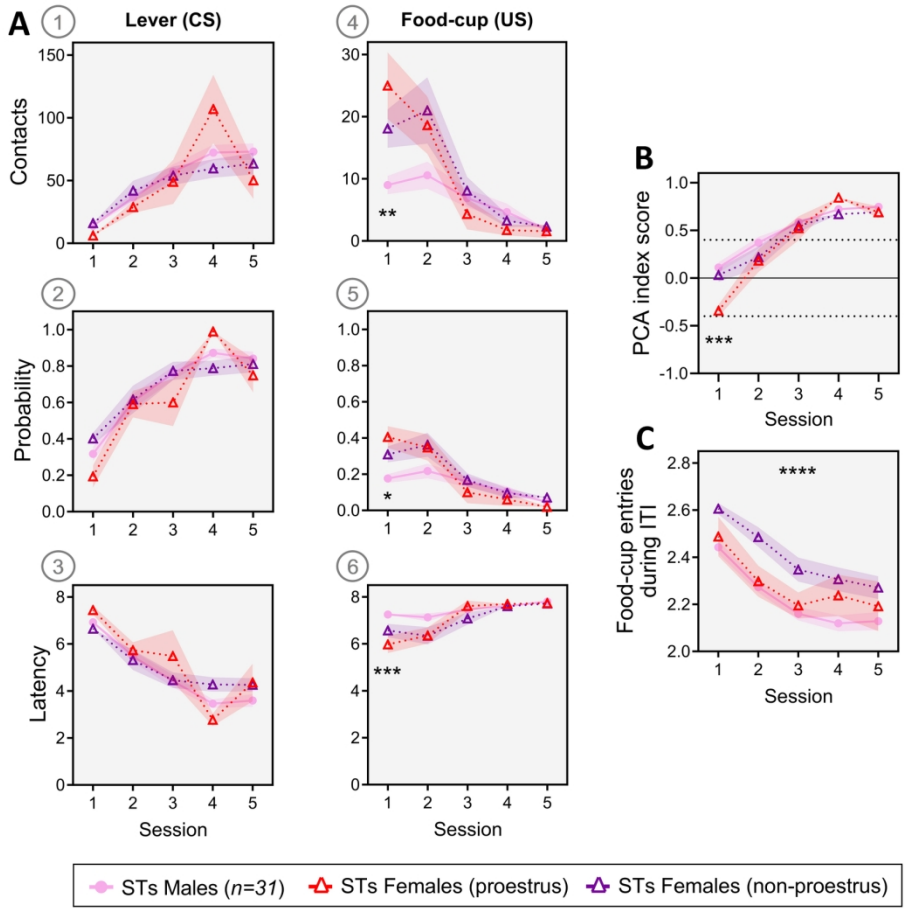


Fig. 8. Conditioned responses across the oestrous cycle for Paired animals.

81x76mm (600 x 600 DPI)

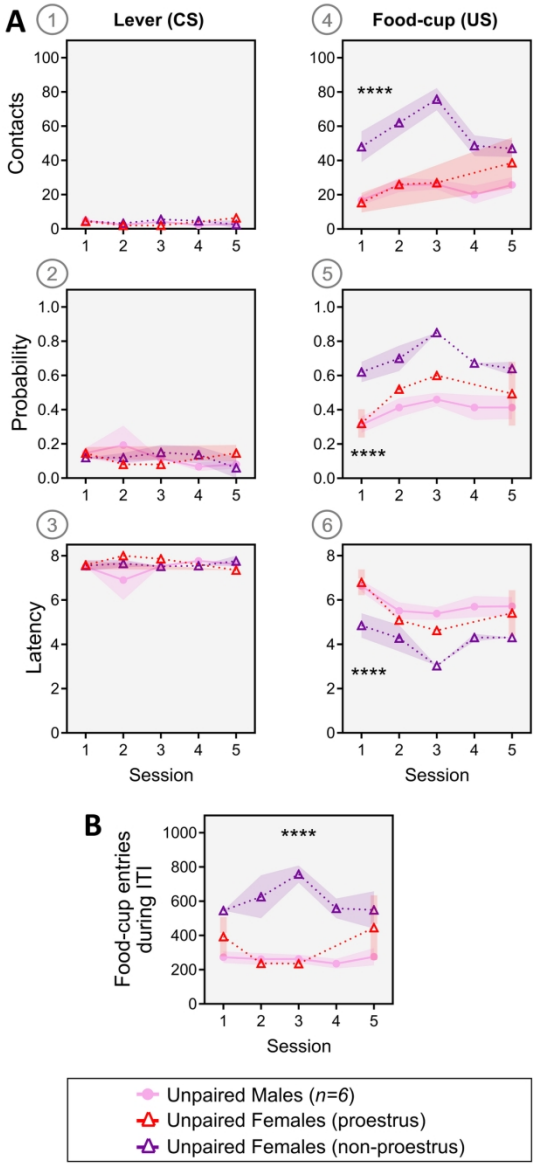
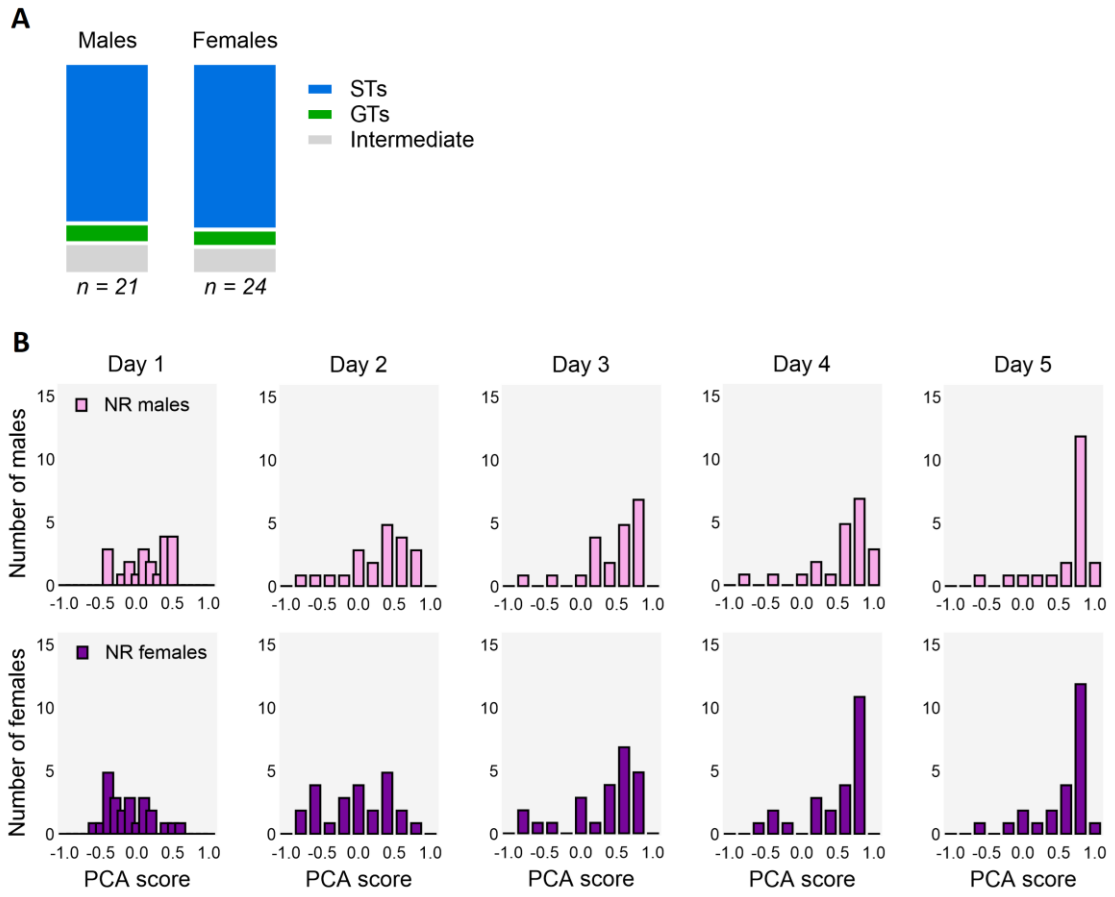


Fig. 9. Responses across the oestrous cycle for Unpaired animals.

54x104mm (600 x 600 DPI)

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.

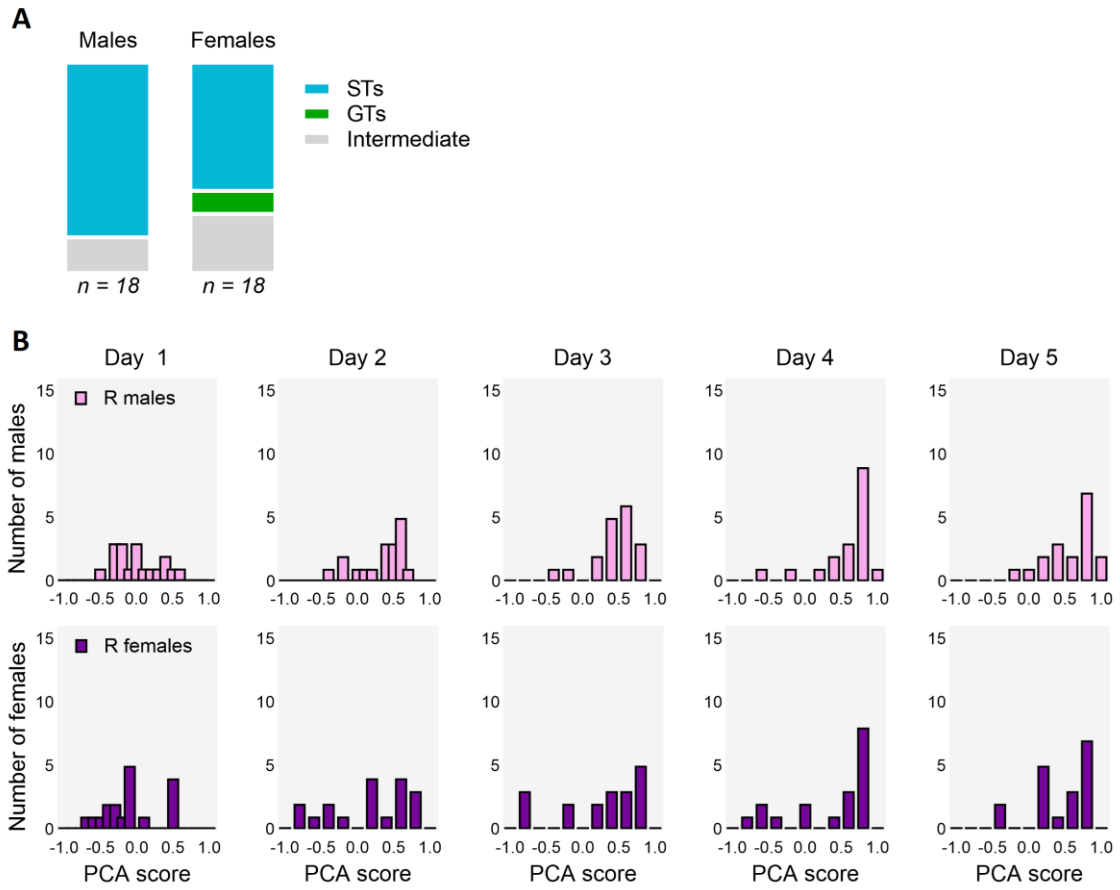


Suppl. Fig. 1. Pavlovian phenotypic repartition of Experiment 1 and evolution across training. (A) Repartition of STs, GTs and intermediate male and female rats from the Paired-NR group (non-rewarded). (B) Evolution of the PCA index score across the five sessions of PCA training in male and female rats from the Paired-NR group.

Male and female rats from the non-rewarded group (Paired-NR-group; Fig. 1) exhibited equivalent individual variation in Pavlovian conditioned approach (Suppl. Fig. 1.A; $\chi^2 = 0.027$, df = 2, p = 0.987). A high majority were classified as STs (77.18% on average), whereas only an average of 8.92% of rats (2 females and 2 males) were categorised as GTs. 13.39% of rats displayed no preference for the lever or for the food magazine ('intermediate'). The distribution of PCA scores across the five conditioning sessions shows a shift from the central range of the score (-0.5 and 0.5) to the highest range for both males and females and illustrates the formation of one main sign-tracking population (Suppl. Fig. 1.B). It is interesting to note that the final propensity emerges earlier in males, for which the ST tendency can already be observed on day 2.

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.

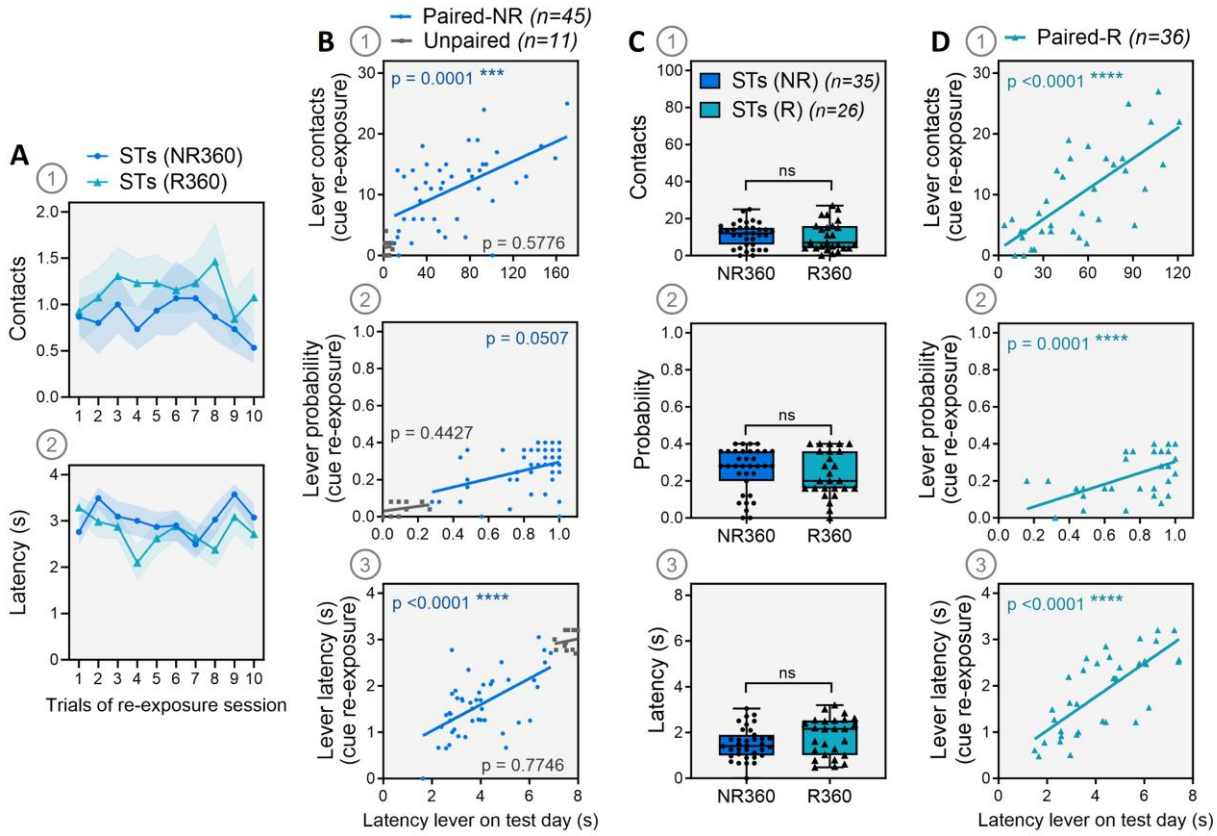


Suppl. Fig. 2. Pavlovian phenotypic repartition of Experiment 2 and evolution across training. (A) Repartition of STs, GTs and intermediate male and female rats from the Paired-R group (rewarded). (B) Evolution of the PCA index score across the five sessions of PCA training in male and female rats from the Paired-R group.

The phenotypic distribution of the Paired-R group (Fig. 1) differed slightly from that of the Paired-NR group (Suppl. Fig. 2.A). Most rats were classified as STs but to a lesser extent, especially in males (males = 55.56%, females = 70.59%). This is due to a higher percentage of animals that did not express an explicit preference towards the cue or the reward location ('intermediate'; males = 33.33%, females = 23.53%). The percentage of GTs was similar to that of Experiment 1 (8.49% on average). Despite these visible differences, a Chi-square test of independence did not reveal any significant effect of sex in the phenotypic repartition ($\chi^2 = 3.115$, df = 2, p = 0.211). The distribution of PCA scores across conditioning sessions still shows the same shift from the central part to the highest range of the score for both sexes, illustrating the development of a core ST population (Suppl. Fig. 2.B). The propensity to sign-track developed earlier in males and can already be observed on day 2.

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.



Suppl. Fig. 3. Rewarded and non-rewarded CS re-exposure. (A) Evolution of lever-directed behaviours across the 10 trials of the unrewarded (*blue circles*) and rewarded (*turquoise triangles*) cue re-exposure, for animals perfused 360 minutes after the test. (B) Correlations between lever-directed CRs of the Paired-NR group (*blue circles*) and the Unpaired group (*grey squares*) during the last training session and the non-rewarded CS re-exposure. (C) Comparison of lever-directed behaviours during the cue re-exposure session between STs of the NR group (non-rewarded cue re-exposure), and STs of the R group (rewarded cue re-exposure). Males and females combined. (D) Correlations between lever-directed behaviours of the R group during the last training session and the rewarded CS re-exposure.

(*** $p \leq 0.001$, **** $p < 0.0001$).

No difference was found in lever contacts (Suppl. Fig. 5.A-1, *blue*; $\chi^2 = 5.480$, df = 9, p = 0.7906) or latency to approach the lever (Suppl. Fig. 5.A-2, *blue*; $\chi^2 = 12.69$, df = 9, p = 0.1772) across the 10 trials of the re-exposure session for NR360 sign-tracking animals. A Friedman test for repeated measures revealed no difference in lever contacts (Suppl. Fig. 5.A-1, *turquoise*; $\chi^2 = 5.162$, df = 9, p = 0.8200) for sign-trackers of the R360 group. A significant difference was found for the latency to first contact the lever in R360 STs (Suppl. Fig. 5.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: $p = 0.0289$).

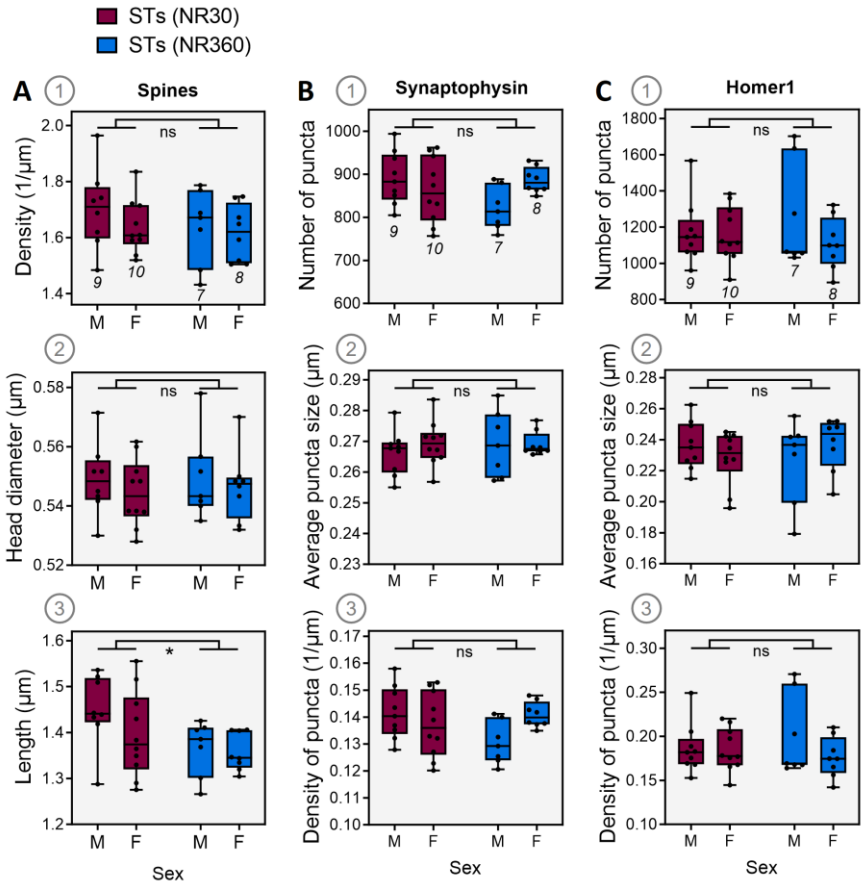
Suppl. Fig. 5.B shows significant correlations in Paired animals between the last training session and the test session for the number of contacts with the lever (Suppl. Fig. 5.B-1; Pearson $r = 0.5447$, $p = 0.0001$) and the latency to first contact the lever (Suppl. Fig. 5.B-3; Pearson $r = 0.5806$, $p < 0.0001$). No relationship was observed in these measures between the last training session and the re-exposure session in Unpaired (Suppl. Fig. 5.B; all Spearman $r < 0.2579$, all $p > 0.4427$).

When comparing STs' conditioned responses between the re-exposure session of Experiment 1 and Experiment 2, no difference was found in lever contacts (Suppl. Fig. 5.C-1; Mann-Whitney: $U = 418.5$, $p = 0.5989$), lever probability (Suppl. Fig. 5.C-2; Mann-Whitney: $U = 4.505$, $p = 0.4719$) or lever latency (Suppl. Fig. 5.C-3; Unpaired t-test: $t=1.616$, $df=59$, $p = 0.1114$).

Correlation analyses of lever-directed behaviours produced by the R360 group indicate that the number of contacts with the lever (Suppl. Fig. 5.D-1; Spearman $r = 0.6928$, $p < 0.0001$), the probability (Suppl. Fig. 5.D-2; Spearman $r = 0.6022$, $p = 0.0001$) and latency (Suppl. Fig. 5.D-3; Spearman $r = 0.7447$, $p < 0.0001$) to first approach the lever during the rewarded re-exposure were significantly correlated to the same measures during the last training session.

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.



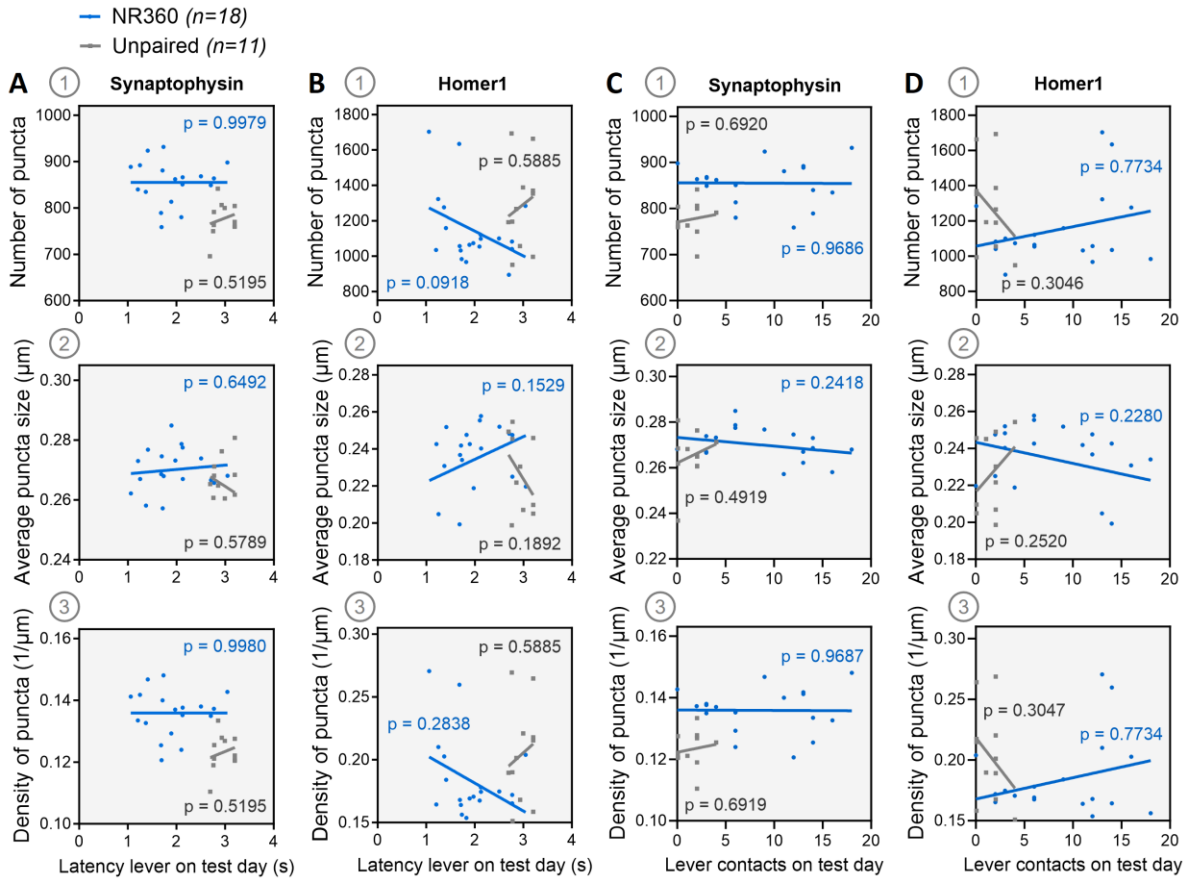
Suppl. Fig. 4. Effect of the time of perfusion on dendritic spine measurements, pre- and postsynaptic activity within the NR group. (A) Spine characteristics in male and female STs of the NR group perfused 30 minutes or 360 minutes after non-rewarded CS re-exposure. (B) Synaptophysin staining in male and female STs of the NR group perfused 30 minutes or 360 minutes after CS re-exposure. (C) Homer1 staining in male and female STs of the NR group perfused 30 minutes or 360 minutes after CS re-exposure. (* $p \leq 0.05$).

A two-way ANOVA comparing spine properties between NR30 (Fig. 1) and NR360 (Fig. 1) male and female rats revealed that ST rats whose brains were fixed 30 minutes after cue re-exposure had longer spines in the NAc core than rats perfused 360 minutes after re-exposure (Suppl. Fig. 3.A; effect of time, $F_{1, 29} = 5.416$, $p = 0.0271$). No difference was found between both groups in the density of spines (effect of time: $F_{1, 29} = 1.056$, $p = 0.3125$) and average diameter (effect of time: $F_{1, 29} = 0.08839$, $p = 0.7684$). No effect of sex was observed (Suppl. Fig. 3.A; effect of sex, all $F_{1, 29} < 1.402$, all $p > 0.2460$). Synaptophysin staining (Suppl. Fig. 3.B; effect of time: all $F_{1, 30} < 1.348$, all $p > 0.2548$; effect of sex: all $F_{1, 30} < 0.8673$, all $p > 0.3591$) and homer1 staining (Suppl. Fig. 3.C; effect of time: all $F_{1, 30} < 0.07838$, all $p > 0.7814$; effect of sex: all $F_{1, 30} < 1.395$, all $p > 0.2469$) in the NAc core of ST from the

NR group were comparable 30 minutes and 360 minutes after re-exposure to the CS and between males and females.

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.



Suppl. Fig. 5. Relationship between pre- and postsynaptic activity and conditioned responses in Paired-NR and Unpaired animals. (A) Correlation between synaptophysin staining and the latency to first contact the CS during the cue re-exposure, in Paired rats of the non-rewarded group perfused 360 minutes after CS re-exposure (NR360; *blue circles*) and Unpaired rats (*grey squares*). (B) Correlation between homer1 staining and the CS latency during the re-exposure session in NR360 animals and Unpaired animals. (C) Correlation between synaptophysin staining and the number of lever presses (CS contacts) during the re-exposure session in NR360 animals and Unpaired animals. (D) Correlation between homer1 staining and the number of lever presses (CS contacts) during the re-exposure session in NR360 animals and Unpaired animals.

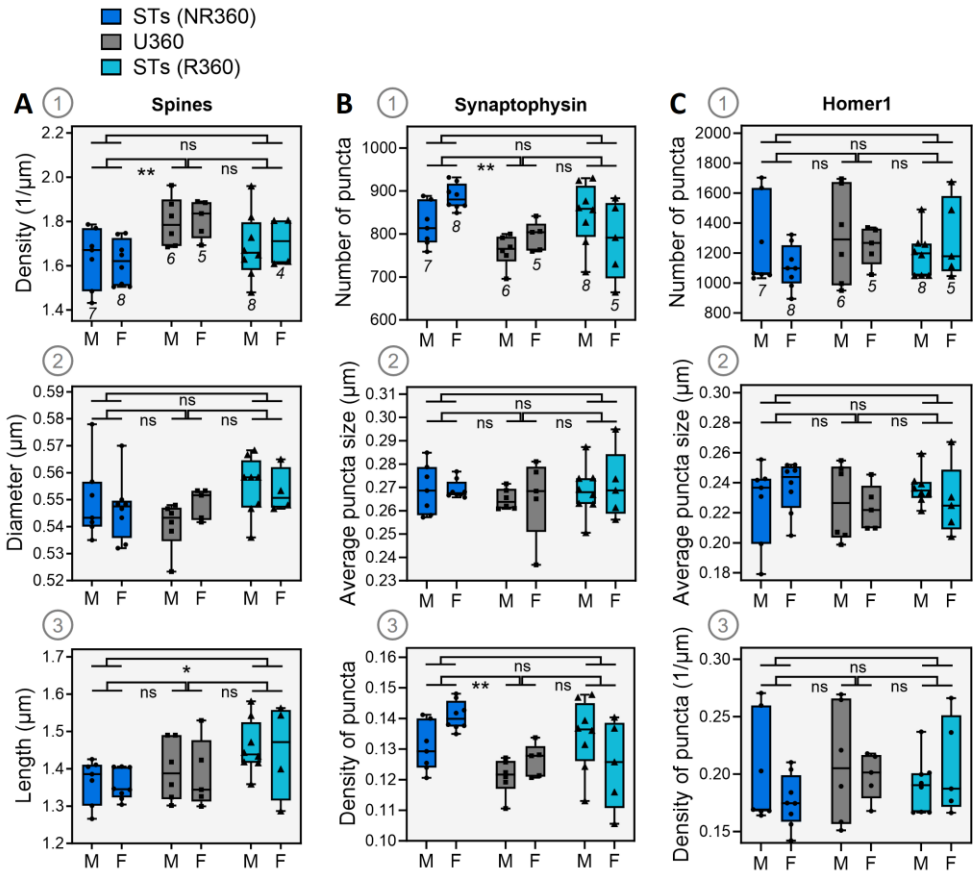
No significant relationship was found between the latency to first contact the lever and synaptophysin staining, neither within the NR360 group that was not exposed to the reward on test day (Suppl. Fig. 4.A; all Pearson $r < 0.1151$, all $p > 0.6492$) nor for the Unpaired group (Suppl. Fig. 4.A; all Pearson $r < 0.2181$, all $p > 0.5195$). Despite visual trends in the number of homer1 puncta (Suppl. Fig. 4.B-1; Pearson $r = -0.3870$, $p = 0.0918$), the size of homer1 puncta (Suppl. Fig. 4.B-2; Spearman $r = 0.3318$, $p = 0.1529$) and density (Suppl. Fig. 4.B-3; Spearman $r = -0.2520$, $p = 0.2838$) of homer1 puncta, staining was not correlated to the latency to first contact the lever on test day in the NR360 group. The

latency was not correlated to homer1 staining in the Unpaired group (Suppl. Fig. 4.B; all Pearson $r < 0.4280$, all $p > 0.1892$).

Considering the number of contacts with the lever during the re-exposure session, no correlation was detected between behaviour and synaptophysin staining in Paired-NR360 rats (Suppl. Fig. 4.C; all Pearson $r < 0.2908$, all $p > 0.2418$) or Unpaired rats (Suppl. Fig. 4.C; all Pearson $r < 0.2323$, all $p > 0.4919$). Lever presses were not related to homer1 staining in NR360 animals (Suppl. Fig. 4.D; all Spearman $r < 0.2822$, all $p > 0.2280$), nor to homer1 staining in Unpaired animals (Suppl. Fig. 4.C; all Pearson $r < 0.3778$, all $p > 0.2520$).

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.



Suppl. Fig. 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 of spines, average spine head diameter and average length of spines 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. (* $p \leq 0.05$, ** $p \leq 0.01$).

Consistent with Figures 4 and 6, a two-way ANOVA confirmed that Unpaired rats had a greater spine density than STs from the NR360 group (effect of training: $F_{2, 32} = 6.868$, $P = 0.0033$; Šídák, $p = 0.0024$) and the rewarded R360 group had longer spines than the unrewarded NR360 group in the NAc core (effect of training: $F_{2, 32} = 4.388$, $p = 0.0207$; Šídák, $p = 0.0161$). 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, $p = 0.1611$).

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

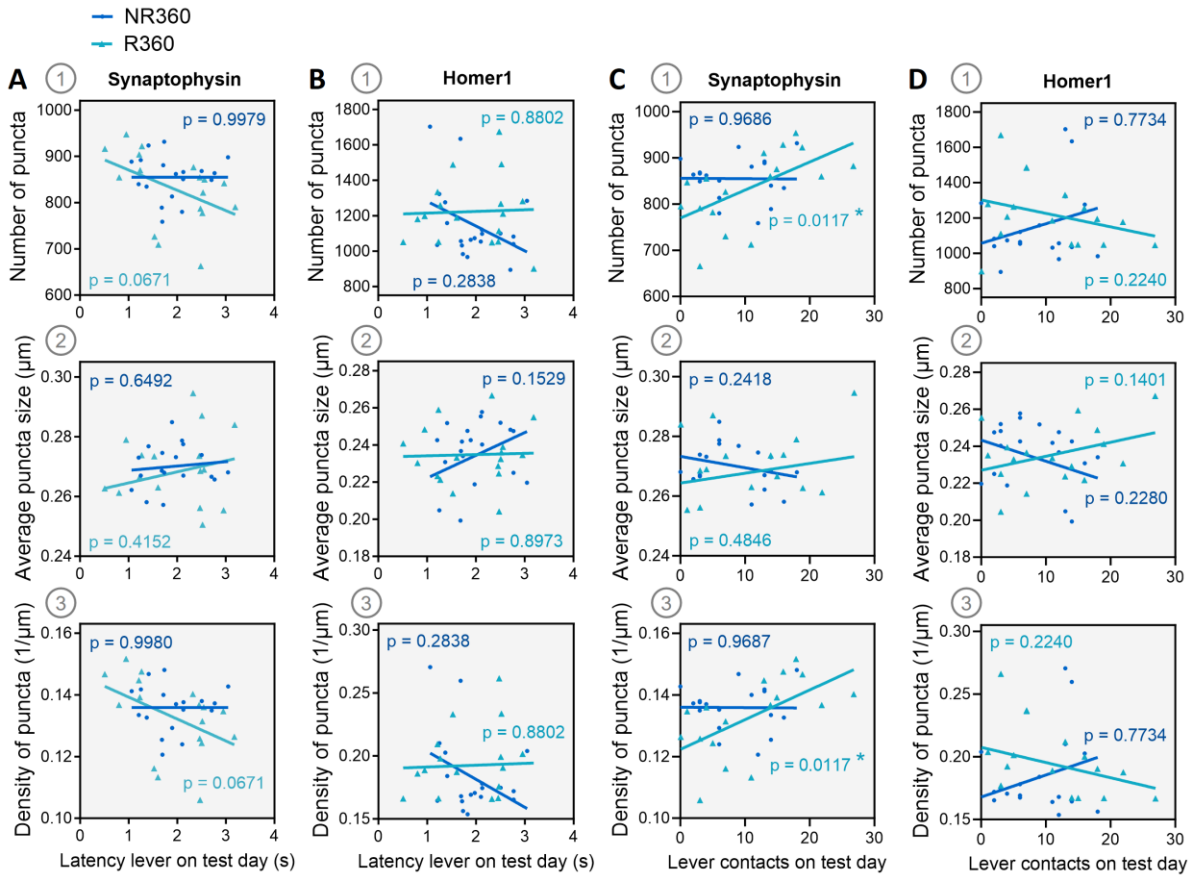
M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.

Contrary to STs from the NR360 group which had more synaptophysin puncta (effect of training: $F_{2, 33} = 5.825$, P = 0.0068; Šídák, p = 0.0053) 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, animals from the R360 group were significantly similar to Unpaired rats in their amount of synaptophysin puncta (Šídák, p = 0.3022), average puncta size (effect of training: $F_{2, 33} = 0.5876$, P = 0.5614) and density of synaptophysin puncta (Šídák, p = 0.2486).

In accordance with Figures 4 and 6, two-way ANOVAs did not find differences in the number of homer1 puncta (effect of training: $F_{2, 33} = 0.6130$, P = 0.5478), average homer1 puncta size (effect of training: $F_{2, 33} = 0.4426$, P = 0.6461) or density of homer1 puncta (effect of training: $F_{2, 33} = 0.6130$, P = 0.5478) between Unpaired animals, STs from the rewarded R360 group and STs from the unrewarded NR360 cohort.

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.



Suppl. Fig. 7. Relationship between pre- and postsynaptic activity and conditioned responses in rewarded and unrewarded Paired animals. (A) Correlation between synaptophysin staining and the latency to first contact the CS during the cue re-exposure, in rats of the rewarded (R360; *turquoise triangles*) group and rats of the non-rewarded group (NR360; *blue circles*) perfused 360 minutes after CS re-exposure. (B) Correlation between homer1 staining and the CS latency during the re-exposure session in rats of the R360 group and the NR360 group. (C) Correlation between synaptophysin staining and the number of lever presses (CS contacts) during the re-exposure session in rats of the R360 group and the NR360 group. (D) Correlation between homer1 staining and the number of lever presses (CS contacts) during the re-exposure session in rats of the R360 group and the NR360 group.

Statistics for the Paired-NR group can be found in Suppl. Fig. 4. In Paired-R animals exposed to the reward during cue re-exposure (Suppl. Fig. 6.A), no significant difference was detected between individuals that approached the lever faster and synaptophysin staining, although positive trends can be observed in the number (Suppl. Fig. 6.A-1; Pearson r = -0.4541, p = 0.0671) and density of puncta (Suppl. Fig. 6.A-3; Pearson r = -0.4541, p = 0.0671). Similarly, no relationship was found between CS

latency and homer1 staining in the R360 group (Suppl. Fig. 6.B; all Pearson $r < 0.03955$, all $p > 0.8802$).

Suppl. Fig. 6.C and 6.D illustrate the relationship between the number of lever presses during the re-exposure session and synaptophysin or homer1 staining. Lever contacts were positively correlated to the number of synaptophysin puncta (Suppl. Fig. 6.C-1; Pearson $r = 0.5951$, $p = 0.0117$) and the density of synaptophysin puncta (Suppl. Fig. 6.C-3; Pearson $r = 0.5951$, $p = 0.0117$) in the Paired-R360 group, but not to the size of synaptophysin puncta (Suppl. Fig. 6.C-2; Pearson $r = 0.1820$, $p = 0.4846$). No relationship was found between lever contacts and homer1 staining in the R360 group (Suppl. Fig. 6.D; Pearson $r < 0.3732$, $p = 0.1401$).

Supplementary data: Cue- and reward-related dendritic and presynaptic plasticity of nucleus accumbens neurons in male and female sign-tracker rats.

M. Colom, I. Kraev, A. K. Stramek, I. B. Loza, C. L. Rostron, C. J. Heath, E. J. Dommett[1], B. F. Singer.

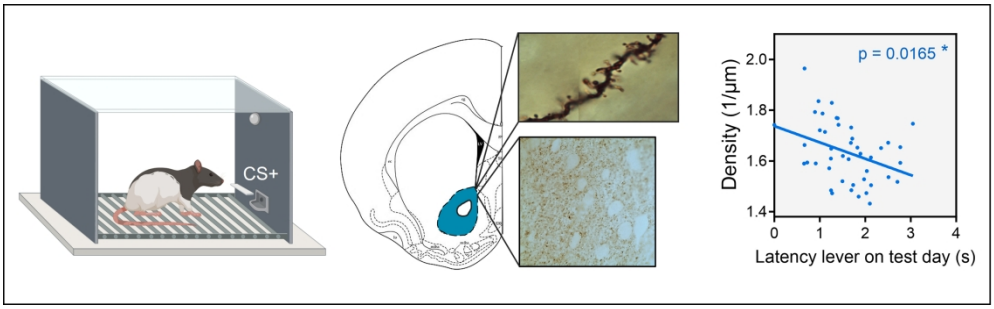
Suppl. Table 1. Quantification of the oestrous cycle repartition in females of Experiments 1 and 2.

	Average length	Proestrus Session 1	Proestrus Session 2	Proestrus Session 3	Proestrus Session 4	Proestrus Session 5
Paired-NR	4 days	7	8	5	3	7
Paired-R	4 days	2	6	3	7	2
Paired (all)	4 days	9	14	8	9	9
Unpaired	4 days	3	2	1	0	3

Suppl. Video 1. Illustration of the 'oestrous dance' exhibited by female rats in proestrus. *Video curtesy of A. K. Stramek and I. B. Loza.* Upon gentle squeezing around the hind legs, a female rat in proestrus arches the back (lordosis), darts, hops, freezes, and vibrates the ears.

Graphical abstract

When attributed with motivational salience, reward-predictive stimuli can drive behaviour and contribute to maladaptive responses. We examined whether sign-tracking conditioned behaviours were associated with specific dendritic spine features, as well as markers of pre- and postsynaptic activity in the nucleus accumbens core of male and female rats. Data suggest that changes in accumbal neuronal plasticity may be related to individual variation in the responsiveness to Pavlovian cues.



When attributed with motivational salience, reward-predictive stimuli can drive behaviour and contribute to maladaptive responses. We examined whether sign-tracking conditioned behaviours were associated with specific dendritic spine features, as well as markers of pre- and postsynaptic activity in the nucleus accumbens core of male and female rats. Data suggest that changes in accumbal neuronal plasticity may be related to individual variation in the responsiveness to Pavlovian cues.

272x81mm (300 x 300 DPI)