Exercise craving potentiates excitatory inputs to ventral tegmental area dopaminergic neurons

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Exercise craving potentiates excitatory inputs to ventral tegmental area dopaminergic neurons

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Abstract

Physical exercise, which can be addictogenic on its own, is considered a therapeutic alternative for drug craving. Exercise might thus share with drugs the ability to strengthen excitatory synapses onto ventral tegmental area (VTA) dopaminergic neurones, as assessed by the ratio of AMPA receptor (AMPAR)-mediated excitatory postsynaptic currents (EPSCs) to NMDA receptor (NMDAR)-mediated EPSCs. As did acute cocaine, amphetamine or Δ^9-tetrahydrocannabinol (THC) pretreatments, an acute 1-h wheel-running session increased the AMPAR/NMDAR ratio in VTA dopaminergic neurones. To dissect the respective influences of wheel-running seeking and performance, mice went through an operant protocol wherein wheel-running was conditioned by nose poking under fixed ratio schedules of reinforcement. Conditioned wheel-running increased the AMPAR/NMDAR ratio to a higher extent than free wheel-running, doing so although running performance was lower in the former paradigm than in the latter. Thus, the cue-reward association, rather than reward consumption, played a major role in this increase. The AMPAR/NMDAR ratio returned to baseline levels in mice that had extinguished the cued-running motivated task, but it increased after a cue-induced reinstatement session. The amplitude of this increase correlated with the intensity of exercise craving, as assessed by individual nose poke scores. Finally, cue-induced reinstatement of running seeking proved insensitive to acute cocaine or THC pretreatments. Our study reveals for the first time that the drive for exercise bears synaptic influences on VTA dopaminergic neurones which are reminiscent of drug actions. Whether these influences play a role in the therapeutic effects of exercise in human drug craving remains to be established.

KEYWORDS

AMPAR/NMDAR ratio, dopamine, motivation, reinstatement, synaptic plasticity, wheel-running

1. INTRODUCTION

Volitional exercise is highly rewarding in laboratory rodents.[1] Thus, mice housed with running wheels cover 5-6 kilometers per day (mostly throughout the dark phase of the dark/light cycle),[2] a performance which doubles in high-runner lines.[3,4] Moreover, conditioned place preference[5] is observed with wheel-running.[6,7] Definitive evidence for the reinforcing value of wheel-running stems from operant conditioning procedures[5] wherein performance of a task, i.e. cued-associated lever pressing or nose poking under fixed (FR), variable and/or progressive (PR) ratio reinforcement schedules, was a running prerequisite.[8-10] As opposed to "free" wheel-running paradigms, in which motivation for running and running performance cannot be disentangled,[11,12] conditioned procedures allow to distinguish each of these two variables.[10,12] This distinction is essential because motivation, but not consumption, of a reward is linked to the activity of ventral tegmental area (VTA) mesocorticolimbic dopaminergic neurones.[13,14] Confirmingly, wheel-running motivation, as assessed during PR sessions,[10] but not free wheel-running performance,[2] is associated with increased firing activity of VTA dopaminergic neurones. Actually, running motivation is tonically controlled by endocannabinoids which likely act by decreasing VTA GABA release, and hence disinhibition of dopaminergic neuronal activities.[10]

Mesocorticolimbic dopaminergic activity mediates the reinforcing properties of natural rewards (e.g. palatable food) and drugs with abuse liability.[14-16] The observation that exercise motivation is associated to the mesocorticolimbic system opens several perspectives. First, practicing exercise can turn into compulsion in vulnerable individuals.[17,18] This compulsive behaviour is either primary (exercise *per se*) or secondary to a pathology, as illustrated in restrictive anorexia.[19,20] Second, exercise is considered an efficient therapeutic alternative for drug craving in humans whilst wheel-running counteracts motivation and/or craving for e.g. cocaine, amphetamine or opiates in rodents.[21,22] These data, thus, underline the need to examine whether exercise shares with drugs the ability to trigger circuit changes key in the development of addictive behaviours.

Long-term impacts of positive reinforcers on synaptic plasticity in the mesocorticolimbic system might contribute to their abuse liability.[23,24] In agreement with the initial observation that systemic cocaine or amphetamine treatments promote transient increases in the responsiveness of VTA dopaminergic neurones to AMPA,[25] these treatments rapidly increased (i.e. within 24 h) the ratio of AMPA receptor (AMPAR)-mediated excitatory postsynaptic currents (EPSCs) to NMDA receptor (NMDAR)-mediated EPSCs, doing so for several days.[26-29] Drug-elicited imbalances between excitatory and inhibitory currents in VTA dopaminergic neurones might even be amplified by concomitant decreases in GABA currents.[30,31] One important limit of these "passive" treatment protocols lies, however, in the failure to link the aforementioned increases in AMPAR/NMDAR ratio with the reinforcing properties of the drug under investigation. By means of cued-reward motivated instrumentals tasks, it was observed that cocaine self-administration increases the AMPAR/NMDAR ratio, an effect fully accounted for by the cue-reward contingency.[32] This increased excitatory input on VTA dopaminergic neurones is maintained at the same level throughout weeks of abstinence.[32] Altogether, these findings indicate that contingent drug self-administration provides a memory trace to VTA glutamatergic-dopaminergic synapses possibly relevant to the craving process.[23] In keeping with the ability of natural rewards to affect synaptic plasticity within the reward circuitry,[24] repeated cued-sucrose (or cued-food) self-administration has also been shown to increase the AMPAR/NMDAR ratio in VTA dopaminergic neurones.[32] Whether another natural reward such as exercise reinforces the glutamatergic tone on VTA dopaminergic neurones is still unknown. If so, it would identify a neurobiological means through which exercise can become compulsive and is therapeutically effective in drug addiction.

The present study measured the AMPAR/NMDAR ratio in VTA dopaminergic neurones of mice given the opportunity to run under FR schedules of reinforcement. To disentangle the respective roles of running motivation and performance on the excitatory input to VTA dopaminergic neurones, these mice were compared to mice provided free running access. Finally, we examined whether exercise craving affects the AMPAR/NMDAR ratio, doing so after an extinction phase followed by a cue-induced reinstatement of running seeking.

2. MATERIALS AND METHODS

2.1 Animals

This study obeyed the French (Décret 2013-118) and European (2010/63/EU) rules on animal experimentation and was approved by the local Ethic Committee with agreement numbers 13649 and 13650 (to F.C.) and A33-063-098 (animal facilities). A first series of experiments used male DAT-Cre/Ai6 mice (aged 7-10 weeks, n = 37) to compare the synaptic effects of a 1-h free (i.e. unconditioned) wheel-running session with those of cocaine, amphetamine or Δ^9-tetrahydrocannabinol (THC). These mice, bred in our animal facilities from initial crossings bewteen B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$/J mice (also referred to as Ai6 mice; The Jackson Laboratory, USA) and B6.SJL-*Slc6a3*$^{tm1.1(cre)Bkmn}$/J mice (also referred to as DAT-Cre mice; The Jackson Laboratory, USA), allowed us to visualise VTA dopaminergic neurones through selective expression of the fluorescent marker ZsGreen1. All other experiments used males (aged 8-12 weeks, n = 73) from a C57BL/6N-derived mouse line bred in our animal facilities, namely the *Cnr1*$^{flox/flox}$ (CB_1-floxed) line. Note that the reinforcing values of wheel-running, the latter being provided free[2] or under FR reinforcement schedules[10] are similar in CB_1-floxed and C57Bl/6N mice.

2.2 Housing

At least one week before experiments, all animals were individually housed (to avoid inter-individual aggression) with food and water *ad libitum* in a thermoregulated room (21-22°C) placed under a partly reversed 12h/12h light/dark cycle (lights off: 9:00-21:00). Mice tested for the synaptic impacts of acute (DAT-Cre/Ai6 mice) and repeated (CB_1-floxed mice) free wheel-running were housed with 25-cm diameter running wheels (IntelliBio, France).[2,12] All mice were tested during the dark phase (between 10:00 and 18:00) of the light/dark cycle.

2.3 Pharmacological treatments

DAT-Cre/Ai6 mice were intraperitoneally injected (10 ml/kg) with either cocaine hydrochloride (15 mg/kg of the salt), amphetamine hydrochloride (10 mg/kg of the salt), THC (1-10 mg/kg) or their respective vehicles (0.9 % NaCl, except for THC which was a mixture of 4 % ethanol and 4 % Cremophor-EL in 0.9% NaCl). All mice, immediately replaced in their housing cages after treatments, were sacrificed 18-24 h later. CB_1-floxed mice were injected either with 15 mg/kg cocaine, 3 mg/kg THC or their respective vehicles (identical doses and procedures as above) 1-2 h after their last extinction session.

2.4 Free (unconditioned) wheel-running

Home cage running wheels were either kept locked (respective control mice) or set 1-h-free once (DAT-Cre/Ai6 mice) or daily for 12 days (CB_1-floxed mice) through their connection to a computer which recorded running performances (ActiviWheel software; IntelliBio, France).[2,12] To ensure that mice tested for the synaptic impact of an acute 1-h run would effectively run, these were given a daily 5-min free habituation run during the 3-4 days that preceded the final 1-h run.

2.5 Conditioned wheel-running

2.5.1 Apparatus

Operant chambers (28 cm x 26 cm x 38 cm; Imetronic, France) were located in a room adjacent to the housing room.[10,12] These chambers, placed inside wooden casings (60 cm x 62 cm x 49 cm), were ventilated to guarantee air circulation and to provide background noise. The rear wall had a hollow with a 20-cm-wheel locked or unlocked (by means of a brake-pad) according to predefined experimental conditions. This wheel was flanked by 2 holes set into the rear wall,

allowing the animal to 'poke' its nose through, with cue-lights located above nose poke ports. An additional light was placed above the wheel, which illuminated while the wheel was unlocked. Nose pokes could be either "active" (simultaneously leading to cue-light illumination above the active port, wheel unlocking and illumination of the wheel) or "inactive" (having no consequence), the left/right allocation of active/inactive ports being counterbalanced between animals. All devices in the operant chambers were linked to the Polywheel software (version 5.2.2; Imetronic, France).

2.5.2 Conditioned wheel-running under fixed ratio reinforcement schedules

The protocol consisted in 1-h daily sessions, as previously described.[10,12] The first day, mice were placed in the chambers, with the light above the unlocked running wheel remaining illuminated during the whole session. The nose poke ports were covered-up by metal pieces and the cue-light above the active port remained off. This phase was performed on 2 consecutive days to habituate the mice to the operant chambers, the wheel and the wheel-light indicating availability of the reward. When learning sessions began (session 1), the wheel locking/unlocking mechanism and the nose poke ports became fully operational. The wheel was unlocked (wheel brake released) following nose pokes the mouse performed in its allocated active port. The other port, although accessible, remained inactive. Learning sessions began with FR1 sessions during which a single active nose poke was sufficient to simultaneously illuminate the cue-light above the port for 10 sec and unlock the running wheel for 1 min under light. When this time period elapsed, the wheel-light extinguished and the brake applied, so that the mouse had to step down from the wheel and execute a further nose poke in order to unlock it again for 1 min. Nose pokes made in the active port while the wheel was already unlocked were without consequence. After completing the FR1 schedule of reinforcement (6 daily sessions), mice were moved on to the FR3 condition (session 7), which was repeated over 3-6 sessions (sessions 9-12), 1-2 mice being daily sacrificed 24 h after their

last FR3 session. As shown under the Results section, the number of active nose pokes did not significantly vary between sessions 9-12.

2.5.3 Cue-induced reinstatement of wheel-running seeking

In another series of experiments, mice were placed under 6 FR1 and 6 FR3 sessions, as described above. Beginning the day after the last FR3 session, mice underwent 1-h daily extinction sessions for 7 consecutive days with active nose poke ports and cue lights being inactive and the wheel remaining locked, as previously described.[10] The day after the last extinction session (session 19), a cue-induced reinstatement session was then performed (session 20). Two minutes after this session began, a single 10-sec lighting of the cue above the active nose poke port appeared. When the animal performed one active nose poke (as for the FR1 schedule) the cue-light was lit again for 5 sec (the wheel remaining locked). Next, three active nose pokes were required (as for the FR3 schedule) to switch on the light, this procedure being then kept constant throughout the session. Whatever the number of active nose pokes required to light the active port, the running wheel remained locked whilst the cue light above the wheel remained inactive.[10]

2.6 Electrophysiological procedures

Twenty four hours after pharmacological or free/conditioned running procedures, mouse cages were light-protected by a black plastic cover before being transferred to the electrophysiological room. Following sacrifice, each brain was rapidly extracted and two horizontal sections of 250 µm containing the VTA were cut in an ice-cold sucrose-based solution (180 mM sucrose, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 11 mM glucose, 0.2 mM $CaCl_2$ and 12 mM $MgCl_2$; 300 mOsm) bubbled with 95% O_2/5 % CO_2 using a vibratome (Leica VT 1200S, Germany) with a double edge stainless steel blade (Electron Microscopy Sciences, USA). Slices were next placed to recover (30 minutes at 34°C followed by 30

minutes at room temperature) in a chamber containing artificial cerebrospinal fluid (aCSF) bubbled with 95% O_2/5 % CO_2. The aCSF composition was: 123 mM NaCl, 2.5 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 11mM glucose, 2.5 mM $CaCl_2$ and 1.3 mM $MgCl_2$ (pH 7.2, 290 mOsm). One slice was then transferred to a recording chamber (Scientifica, USA) maintained at 32-34°C and continuously perfused with aCSF (bubbled with 95% O_2/5 % CO_2) at a flow rate of 2 ml/min. Cells were visualised with an upright microscope with infrared illumination (Olympus U-CMAD3, France). For DAT-Cre/Ai6 mice, a LED illumination system (CoolLed PE excitation system) was used to identify fluorescent dopaminergic neurons from the latero-posterior VTA (i.e. medial to the medial terminal nucleus of the accessory optic tract). The stimulating electrode, a bipolar stainless steel electrode (FHC, USA), was placed 100 µm rostrally to the recording area. Recording electrodes were pulled on the day of the experiment (Sutter Instruments, USA) from borosilicate glass and filled with an internal solution containing: 117 mM Cs methansulfonic acid, 20 mM HEPES, 0.4 mM EGTA, 2.8 mM NaCl, 5 mM TEA-Cl, 2.5 mM Mg_2ATP, and 0.25 mM Mg_2GTP (pH 7.2–7.4, 280–290 mOsm). All neurones were filled with biocytin 0.2%, biocytin-positive neurons of CB_1-floxed mice being then confirmed by immunocytochemistry to be positive for tyrosine hydroxylase (TH). The final electrode resistance was 3-5 MΩ. Both stimulating and recording electrodes were lowered to the slice by two manipulators (SM-8, Luigs and Neumann, USA). Whole-cell recordings of EPSCs were performed in voltage clamp mode (Vm = - 60 mV) after series resistance was stabilised (typically 10–25 MΩ). Series and input resistance were automatically monitored on-line. Data were filtered at 2 kHz, digitised at 10 kHz, and collected on-line with an acquisition software (pClamp 8.2; Molecular Devices, USA). In all cases, VTA neurons were recorded under differential interference contrast. The presence of a clear I_h current[33] was checked immediately after break-in, using a series of incremental 20 mV-hyperpolarizing steps from a holding potential of - 60 mV to -140 mV. The stimulating electrode was used to electrically stimulate excitatory afferents at 0.1 Hz (50 µsec stimulation at 10-50 V) that were pharmacologically isolated by adding picrotoxin (100 µM in 0.01% ethanol; Sigma, France) to the aCSF in order to block $GABA_A$-mediated currents. As mentioned above, the AMPAR/NMDAR ratio was

calculated to assess the excitatory synaptic strength in VTA dopaminergic neurones. To do so, the peak amplitude of 15 evoked EPSCs recorded at + 40 mV was measured before and after application of the NMDAR antagonist D-AP5 (50 µM in milliQ water; Tocris, UK). NMDAR EPSCs were obtained by subtracting the average EPSC in the presence of D-AP5 from that recorded in its absence.[26] Each slice was exposed only once to D-AP5. One-three neurones were recorded per mouse.

2.7 Immunocytochemistry

After recordings, slices containing neurons labeled with biocytin were directly fixed in 4% paraformaldehyde for 24 h. TH-immunostaining was performed using mouse anti-TH (1:1000; Merck Millipore, Germany) in slices from CB_1-floxed mice and/or streptavidin Texas Red (1:500; Perkin Elmer, USA) in slices from DAT-Cre/Ai6 and CB_1-floxed mice to reveal biocytin labeling. The secondary antibody used for the TH-immunostaining was Alexa Fluor 488-donkey anti-mouse (1:500; Carlsbad, USA). Image acquisition was performed with a Leica DM4000 B LED using a 10 x/0.30 objective. Images were processed by a Leica LAS AF software.

2.8 Statistics

Data presented under curves are shown as mean ± SEM whilst all other data are shown as box-and-whisker plots with center lines indicating medians, box edges representing the 25th-to-75th percentiles, and whiskers reaching the smallest and the largest values. Electrophysiological data are provided as averages from all neurones recorded within each mouse. Data were compared with either a Mann–Whitney test (2-group comparisons) or a Kruskal–Wallis analysis of variance (multiple-group comparisons). If the Kruskal-Wallis analysis proved significant, *post hoc* comparisons using a Mann-Whitney test were then

performed. Significance of the linear relationships between AMPAR/NMDAR ratios and either free wheel running distance or active nose poke performance was assessed by one-way analyses of variances for regression lines. All analyses were achieved using GB-Stat software (version 10.0; Dynamic Microsystems Inc., CA, USA).

3. RESULTS

3.1 Acute free wheel-running potentiates excitatory inputs to VTA dopaminergic neurones

We first ensured that drugs potentiated the excitatory input onto VTA dopaminergic neurones in late adolescent-to-young adult mice tested during the active phase of the light/dark cycle. Pretreatments with 15 mg/kg cocaine or 10 mg/kg amphetamine 24 h beforehand actually increased the AMPAR/NMDAR ratio in VTA dopaminergic neurones (Figures 1A, 1B). This was also true for a 3 mg/kg dose (but not 1 and 10 mg/kg doses; data not shown) of THC, the primary psychoactive component of cannabis (Figures 1C, 1D). Based on these results, we next tested the impact of a single 1-h bout of free (i.e. unconditioned) wheel-running on the strength of excitatory inputs to VTA dopaminergic neurones, as measured 24 h later. Free wheel-running potentiated the excitatory synapses onto VTA dopaminergic neurones (+ 66 ± 21 %; Figures 1E, 1F), albeit to a lower extent than amphetamine (+ 238 ± 31 %; P = 0.0025 by a Mann-Whitney test following a Kruskal-Wallis analysis), but not cocaine or THC. This potentiating effect raised the question of the contribution of running performance *per se*. A correlation analysis of the relationship between the AMPAR/NMDAR ratio and running performance, as assessed by running distance, revealed that free wheel-running potentiation of excitatory input to VTA dopaminergic neurones was independent from running performance (Figure 1G).

3.2 Conditioned wheel-running potentiates excitatory inputs to VTA dopaminergic neurones: comparison with free wheel-running

To measure the consequences of a cue-induced reinstatement session of wheel-running seeking on excitatory inputs onto VTA dopaminergic neurones, we first ensured that conditioned wheel-running potentiated these inputs. Thus, we used an operant conditioning

paradigm wherein mice ran under FR reinforcement schedules (Figure 2A). Following FR1 trainings, mice were tested throughout the last three FR3 sessions when nose poke and wheel-running performances were stabilised (Figure 2B). As shown in Figures 2C and 2D, the exposure to FR3 reinforcement schedules increased the AMPAR/NMDAR ratio compared to their controls, i.e. mice daily transferred in operant chambers with inactive lights/nose poke ports and a locked wheel. The intrinsic contribution of wheel-running performance in this increase was analysed by performing a parallel experiment with mice housed with running wheels (Figure 2E) but allowed to freely run 1 h/day for a similar number of sessions (i.e., 12 sessions; Figure 2F). Although a 1-h free run increased the AMPAR/NMDAR ratio (Figures 2G, 2H), it did so with a much lower amplitude (+ 102 ± 27 % increase) than under the aforementioned FR3 wheel-running session (+ 230 ± 17 %; P = 0.0079 by a Mann-Whitney test). Taken with this result, the observation that the running performance of conditioned mice was much lower than that of free running mice (P = 0.0079; Figures 2D, 2H) indicated that the potentiation of the excitatory synapses onto VTA dopaminergic neurones in conditioned runners was only partly accounted for by running performance.

3.3 Relationship between the intensity of craving for wheel-running and the potentiation of excitatory inputs to VTA dopaminergic neurones

To examine whether craving for wheel-running, as assessed by a cue-induced reinstatement session of running seeking, affects the excitatory input on VTA dopaminergic neurones, mice were first trained under FR1 and FR3 reinforcement schedules (Figure 3A). Mice were then exposed to 7 extinction sessions during which active nose poke responses initially increased above those measured during FR3 sessions (141 ± 12 vs 63 ± 7; P < 0.001) before rapidly declining as anticipated for an extinction procedure (Figure 3B). When exposed to a cue-induced reinstatement session 24 h after the last extinction session, mice increased their numbers of active nose pokes though with variability (Figure 3D). Accordingly, a trend for increased AMPAR/NMDAR ratios (+ 109 %) was observed in this mouse group (Figure 3C,

3D). These heterogeneous responses prompted us to perform a correlation analysis between these variables. This correlation was positive (Figure 3E), suggesting that the potentiation of excitatory inputs was associated to the intensity of exercise craving.

3.4 Pretreatments with cocaine or THC do not affect craving for wheel-running

In keeping with the effects of (i) cocaine, amphetamine or THC and (ii) a cue-induced reinstatement session of wheel-running seeking on glutamatergic plasticity in VTA dopaminergic neurones, we wondered whether drug-induced synaptic changes affect craving for wheel-running. Two series of experiments were thus designed wherein mice were acutely injected either with 15 mg/kg cocaine or its vehicle (Figures 4A, 4B) or with 3 mg/kg THC or its vehicle (Figures 4D, 4E) 1-2 h after the termination of the last extinction sessions. These mice were then examined 24 h later for their active nose poke responses during a cue-induced reinstatement session of wheel-running seeking. Although reinstatement was effective in both series of experiments ($p = 0.0002$ and $p = 0.0016$ for the respective differences with the last extinction scores; Figures 4C, 4F), it proved insensitive to prior treatment with either cocaine (Figure 4C) or THC (Figure 4F).

4. DISCUSSION

Volkow et al. (2017) recently mentioned the need to identify the mechanisms underlying "… the therapeutic benefits of physical activity in addiction".[16] The present study reports that, as drugs with abuse liability, exercise practice (modelled by wheel-running) potentiates the excitatory input onto VTA dopaminergic neurones. This effect is reinforced by prior cue-exercise pairings, exercise performance playing a minor role therein. Lastly, exercise craving after an extinction period increases the AMPAR/NMDAR ratio in VTA dopaminergic neurones with a magnitude associated to craving intensity.

Studies on the impacts of rewards on AMPAR/NMDAR ratios have been mostly carried out with rats or post-weaning mice during the light (i.e. the inactive) phase of the light/dark cycle. Because rodents run during the dark phase of the diurnal cycle,[2] our operant conditioning procedures have been set during that phase.[10,12] Past evidence for circadian regulation of VTA dopaminergic activities[34,35] prompted us to confirm first that *in vivo* cocaine or amphetamine treatments increase the AMPAR/NMDAR ratio under our environmental conditions. For comparison, we used doses similar to those used in mouse studies[26,27,29,30] although these can be considered high with respect to their behavioural effects. The positive effects of cocaine or amphetamine treatments on the AMPAR/NMDAR ratio are shared by other drugs with abuse liability,[27] strengthening the hypothesis that drug addiction involves, at least during its early phases (but see Engblom et al.[36]), a potentiation of excitatory synapses onto VTA dopaminergic neurones.[23] On the other hand, the effects of THC on AMPAR/NMDAR ratios have not been documented so far. We provide evidence for a stimulatory impact of THC - albeit at one dose only - on excitatory synapses onto VTA dopaminergic neurones. Because THC is thought to increase the activity of VTA dopaminergic neurones through a disinhibition of their GABAergic afferences[37-39] (but see Good and Lupica[40]), the mechanism through which THC increases the AMPAR/NMDAR ratio remains elusive.

The main goal of this study was to evaluate whether exercise potentiates the excitatory inputs onto VTA dopaminergic neurones. In a first series of experiments, wheel-running was

provided freely and acutely for comparison with drug treatments. Such a paradigm increased the AMPAR/NMDAR ratio, doing so independently from running performance. This already suggested that the exercise-induced strengthening of the excitatory input to VTA dopaminergic neurones was accounted for by the reinforcing value of the reward, rather than by its consumption. The LTP-like consequences of the initial exposure to an unknown reward might be one mechanism through which this reward, especially when highly reinforcing, leaves a memory trace at excitatory synapses onto VTA dopaminergic neurones. One issue concerns the structures to which these dopaminergic neurones project. Taken with the observation that recordings were performed in the lateral part of the VTA (where DAT mRNA are highly expressed),[41] the presence of an Ih as a prerequisite for recordings and the sensitivity of these neurones to rewards are indices suggesting that these neurones project to the nucleus accumbens.[42]

Cocaine increases extracellular glutamate levels in the VTA only if provided through a cued-reward motivated task.[43] In agreement, the latter procedure has then been shown to be the most effective means through which cocaine increases in the long-term the AMPAR/NMDAR ratio in VTA dopaminergic neurones.[32] This need for an association between a context cue and availability of cocaine suggests that drug-induced increases in the AMPAR/NMDAR ratio are somewhat related to their early addictogenic properties.[23] These results led us to examine whether cue-conditioned wheel-running increases the AMPAR/NMDAR ratio. Indeed, mice that underwent wheel-running under an FR3 reinforcement schedule displayed such an increase. It is however unknown if this increase was accounted for by the last operant session or by the whole operant protocol. In this context, it is noteworthy that rats learning a cued-sucrose association display an increased AMPAR/NMDAR ratio until stabilisation of their daily performance, but not thereafter.[44] This result might be due to the short latency before electrophysiological assays because another study reported long-lasting increases in AMPAR/NMDAR ratios after stabilisation of sucrose self-administration.[32] Our results are in line with the latter observations as our mice all had stabilised their operant performances before undergoing the electrophysiological procedures.

The additional finding that these mice had also stabilised their running performances beforehand suggests that running *per se* might have contributed to the potentiation of the excitatory synapses onto VTA dopaminergic neurones. Although running performance and the magnitude of the AMPAR/NMDAR ratio following an acute running session were not related (see above), one cannot exclude a role for repeated running performance. Indeed, repeated free wheel-running, albeit for a longer duration (i.e. 4-6 weeks), increases the number of tyrosine hydroxylase transcripts in VTA dopaminergic neurones and ΔFosB immunoreactivity in the nucleus accumbens.[45] Our analyses of AMPAR/NMDAR ratios in mice that were allowed to run freely and repeatedly - for a number of sessions equivalent to that used in the operant experiments – suggest that running performance might have a positive influence on these ratios. However, the comparison between running performances on the one hand, and AMPAR/NMDAR ratios on the other hand, under unconditioned (free) and conditioned wheel-running sessions suggests a prominent role of the cue-running association (reward seeking) over running performance (reward consumption). It remains to be established if this rule applies to other natural rewards.

Whether elicited by a cue, a context, drug priming, or stress, reinstatement of drug seeking following a period of extinction (of the drug-reinforced behaviour) in laboratory animals is thought to model human vulnerability to drug relapse.[46,47] Therefore, this model has gained interest in the quest for neurobiological underpinnings of drug craving and relapse. As an illustration, distinct receptor subunits of AMPAR and NMDAR in VTA dopaminergic neurones have been shown to modulate cocaine seeking.[36] To date, only one study has examined the impact of cue-induced reinstatement of reward seeking on the excitatory input to VTA dopaminergic neurones.[32] This study reported that the AMPAR/NMDAR ratio was increased by cue-induced reinstatement of cocaine seeking; however, the magnitude of that increase was similar to that observed during the extinction period that preceded reinstatement.[32] This suggests that cocaine craving does not differ from extinction with regard to the memory traces left through VTA dopaminergic neurones. However, the AMPAR/NMDAR ratio was measured immediately after reinstatement,[32] questioning the magnitude of this ratio at later times (but

see Mameli et al.[29]). As opposed to drug seeking, whether seeking a natural reward affects the AMPAR/NMDAR ratio has not been documented. We first showed that extinction of wheel-running seeking diminished the AMPAR/NMDAR ratio, compared with that measured in FR3 sessions. It is unknown if this observation was accounted for by the extinction procedure and/or by the time period elapsing between FR and extinction sessions. The inclusion of a forced abstinence protocol wherein mice stay in their home cages after the last FR session would help solve this uncertainty. We next showed that the exposure to a cue-induced reinstatement session of wheel-running seeking strengthened excitatory synapses onto VTA dopaminergic neurones. Furthermore, the magnitude of that strengthening was related to the intensity of wheel-running seeking. Although we acknowledge the limits of our animal model of human exercise, this study opens the route for future investigations on neurobiological underpinnings of compulsive exercise. Only gambling is recognised as a behavioural addiction by the most recent version of the Diagnostic and Statistical Manual of Mental Disorders (DSM 5);[48] however, there is an increasing number of reports indicating that exercise, especially running, might become compulsive.[17,18] Because exercise compulsion is documented in female patients suffering restrictive anorexia,[19,20] our study further indicates the need to study the respective influences of animal gender and food restriction on the exercise-elicited potentiation of excitatory synapses onto VTA dopaminergic neurones.

Exercise positively impacts drug motivation/craving, whether alcohol and nicotine (humans), or cocaine, amphetamine and opiates (rodents) are considered.[21,22] Conversely, alcohol and nicotine (i.e. legal drugs) are the privileged compensations for exercise craving in human subjects who withholded exercise.[49] One explanation for these drug-exercise interactions is that their respective drives lie on common neurobiological pathways. Future works should, thus, examine if (i) wheel-running occludes the drug-elicited increase in the AMPAR/NMDAR ratio in VTA dopaminergic neurones, and (ii) craving for wheel-running is decreased by prior drug self-administration. With respect to the latter issue, only one study has examined the delayed (i.e. 24 h) effects of a drug with abuse liability (morphine through fluid intake) on the reinforcing value of wheel-running.[50] Excepted for high doses which decreased

the reinforcing value of wheel-running, morphine proved ineffective. However, the drive for wheel-running increased immediately upon morphine withdrawal. In this context, we examined whether cocaine and THC (at doses increasing the AMPAR/NMDAR ratio 24 h thereafter) affected cue-induced reinstatement of wheel-running seeking. The results suggested that an increase in the AMPAR/NMDAR ratio prior to the cue-induced reinstatement session does not diminish craving for wheel-running. Besides the obvious restrictions due to our drug treatment protocol, whether its duration (acute vs repeated) or its procedure (passive vs self-administration) are concerned, this negative result might be accounted for by the informative limits of the AMPAR/NMDAR ratio. Thus, different stimuli might lead to increased values of that index although they do so through different mechanisms. As an illustration, corticosterone release mediates acute stress-elicited, but not cocaine-elicited, increases in the AMPAR/NMDAR ratio at VTA dopaminergic neurones.[27] Accordingly, we cannot reject the hypothesis that drug addiction and exercise craving occur through independent mechanisms.

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AUTHOR CONTRIBUTIONS

M.C.M., I.H., F.G., M.M., G.M., and F.C. designed the experiments. M.C.M., I.H., E.M., B.R., C.S. and F.C. performed the experiments. M.C.M., I.H., E.M., and F.C. analysed the data. F.C. wrote the initial manuscript and all authors reviewed its content and approved its final version.

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LEGENDS TO THE FIGURES

FIGURE 1 A 1-h free wheel-running session increases the AMPAR/NMDAR ratio independently from running performance. A, Sample traces of AMPAR- and NMDAR-mediated postsynaptic currents recorded from VTA dopaminergic neurones of mice injected with saline, 15 mg/kg cocaine or 10 mg/kg amphetamine 24 h beforehand. B, AMPAR/NMDAR ratio from mice injected with saline (7 neurones), cocaine (7 neurones) or amphetamine (8 neurones). C, Sample traces of AMPAR- and NMDAR-mediated postsynaptic currents recorded from VTA dopaminergic neurones of mice injected with either vehicle or 3 mg/kg THC 24 h beforehand. D, AMPAR/NMDAR ratio from mice injected with vehicle (7 neurones) or THC (9 neurones). E, Free (unconditioned) running apparatus in the home cage and sample traces of AMPAR- and NMDAR-mediated postsynaptic currents recorded from VTA dopaminergic neurones of mice housed with either a locked wheel or a wheel unlocked for 1 h (traces recorded 24 h after running). F, AMPAR/NMDAR ratio (left axis) and running distances (right axis) in control mice (locked wheel; 8 neurones) and in 1-h running mice (12 neurones). G, Lack of relationship between 1-h wheel-running performances and the amplitudes of AMPAR/NMDAR ratio measured 24 h thereafter. All data are shown as box-and-whisker plots with center lines as medians, box edges as 25th-to-75th percentiles, and whiskers as the extreme individual values. Single values (circles) represent the averaged data from the number of neurones indicated above. * $P < 0.05$ and ** $P < 0.01$ for the respective impacts of the drugs and wheel-running on the AMPAR/NMDAR ratio. Scale bars: 20 pA,10 ms.

FIGURE 2 Conditioned wheel-running increases the AMPAR/NMDAR ratio to a larger extent than does free wheel-running. A, Operant chamber set-up with active/inactive nose poke ports (NP) allowing 1-min unlockings of the running wheel. B, Active and inactive nose pokes (left axis) and running distances (right axis) under FR1 and FR3 schedules of reinforcement (5 mice up to session 9, and 4 mice to 1 mouse for sessions 10, 11, and 12, respectively). C, Sample traces of AMPAR- and NMDAR-mediated postsynaptic currents

recorded from VTA dopaminergic neurones of a control mouse (daily transfer to the operant chambers with no possibility to run) and of a mouse exposed to a FR3 reinforcement schedule 24 h beforehand. D, AMPAR/NMDAR ratio (left axis) and running distances (right axis) in control mice (8 neurones) and in mice exposed to an FR3 session (7 neurones). E, Free (unconditioned) running apparatus in the home cage. F, Running distances in mice freely allowed to run daily for 1 h in their home cages. G, Sample traces of AMPAR- and NMDAR-mediated postsynaptic currents recorded from VTA dopaminergic neurones of mice housed with either a locked wheel or a wheel daily unlocked for 1 h (traces recorded 24 h after running). H, AMPAR/NMDAR ratio (left axis) and running distances (right axis) in control mice (locked wheel; 6 neurones) and in mice allowed free running 1 h per day (6 neurones). Except for the data shown under panels B and D, which represent means ± SEM, all data are shown as box-and-whisker plots with center lines as medians, box edges as 25th-to-75th percentiles, and whiskers as the extreme individual values. Single values (circles) represent the averaged data from the number of neurones indicated above. * $P < 0.05$ and ** $P < 0.01$ for the respective impacts of conditioned and free wheel-running on the AMPAR/NMDAR ratio. $^{++}$ $P < 0.01$ for the relative (percent change in AMPAR/NMDAR; see text) and absolute (distance) differences with conditioned running. Scale bars: 20 pA,10 ms.

FIGURE 3 The impact of the cue-induced reinstatement of wheel-running seeking on the AMPAR/NMDAR ratio is associated to the intensity of exercise craving. A, Active and inactive nose pokes for wheel-running under FR1 and FR3 schedules of reinforcement (n = 8). B, Active and inactive nose pokes for wheel-running during extinction sessions (n = 8). C, Sample traces of AMPAR- and NMDAR-mediated postsynaptic currents recorded from VTA dopaminergic neurones of mice exposed respectively to the last extinction session (session 19) and to the cue-induced reinstatement session (session 20) 24 h beforehand. D, Numbers of active nose pokes (left axis) and AMPAR/NMDAR ratio (right axis) during the last extinction session (7 neurones) and during the cue-induced reinstatement session (10 neurones). E, Positive correlation between the individual number of active nose pokes performed during the cue-

induced reinstatement session and the respective amplitude of the AMPAR/NMDAR ratio measured 24 h thereafter. Data are shown either as mean ± SEM (panels A and B) or as box-and-whisker plots with center lines as medians, box edges as 25th-to-75th percentiles, and whiskers as the extreme individual values. Single values (circles) represent the averaged data from the number of neurones indicated above. ** $P < 0.01$ for the difference between the last extinction session and the cue-induced reinstatement session; *** $P < 0.001$ for the difference between the first extinction session and the last FR3 session. Scale bars: 20 pA,10 ms.

FIGURE 4 Cocaine or THC pretreatments do not affect cue-induced reinstatement of wheel-running seeking. A, Active and inactive nose pokes for wheel-running under FR1 and FR3 schedules of reinforcement (n = 19). B, Active and inactive nose pokes for wheel-running during extinction sessions before saline or 15 mg/kg cocaine treatments (n = 19). C, Numbers of active nose pokes during a cue-induced reinstatement session of wheel-running seeking (session 20) in mice pretreated 24 h beforehand with saline (n = 9) or cocaine (n = 10). D, Active and inactive nose pokes for wheel-running under FR1 and FR3 schedules of reinforcement (n = 20). E, Active and inactive nose pokes for wheel-running during extinction sessions before vehicle or 3 mg/kg THC treatments (n = 20). F, Numbers of active nose pokes during a cue-induced reinstatement session of wheel-running seeking in mice pretreated 24 h beforehand with vehicle (n = 10) or THC (n = 10). Except for the data shown under panels C and F, all data represent means ± SEM. Data shown under panels C and F are shown as box-and-whisker plots with center lines as medians, box edges as 25th-to-75th percentiles, and whiskers as the extreme individual values.** $P < 0.01$ and *** $P < 0.001$ for the respective differences with the last extinction sessions.

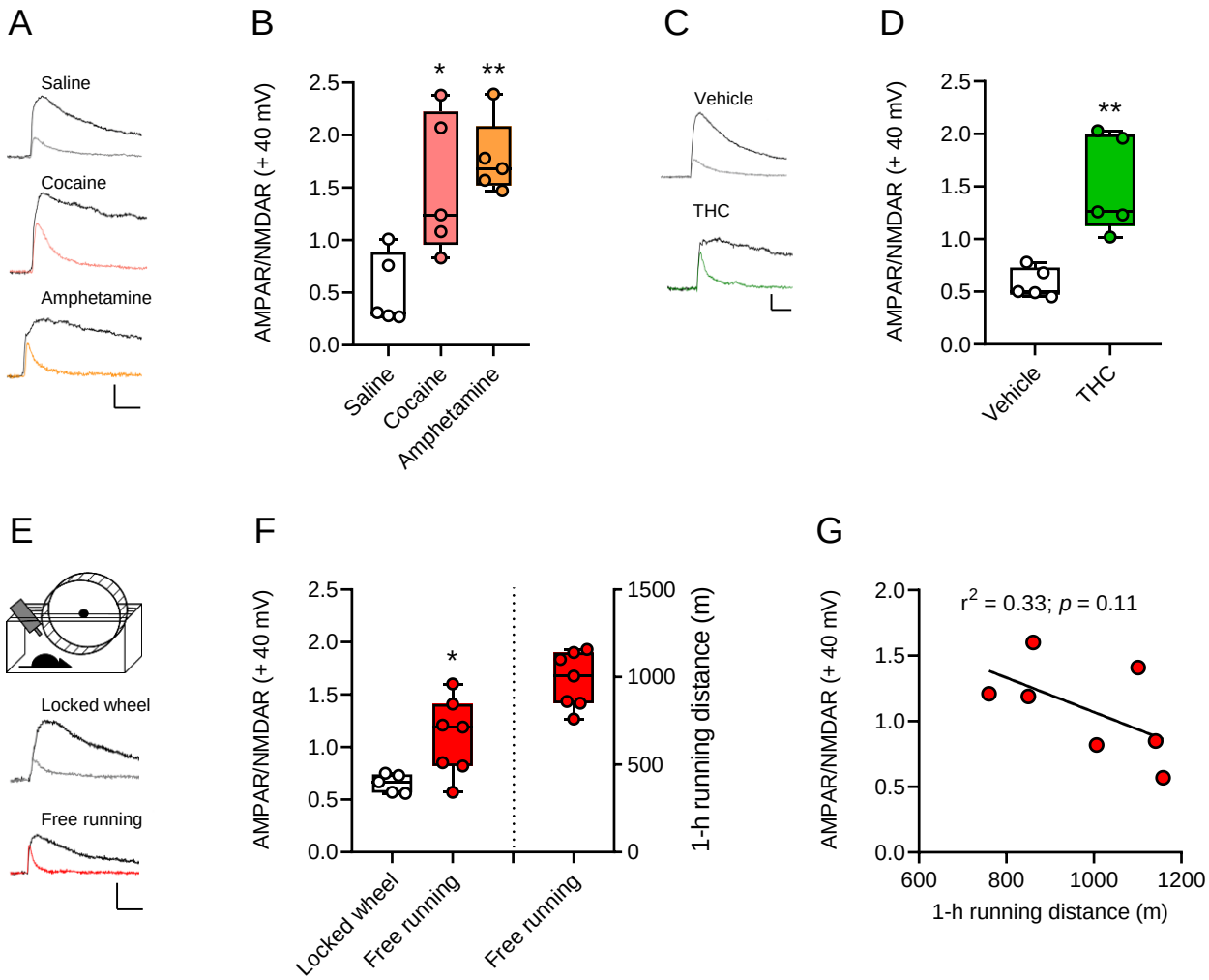
A
Saline
Cocaine
Amphetamine
B
AMPAR/NMDAR (+ 40 mV)
2.5
2.0
1.5
1.0
0.5
0.0
*
**
Saline
Cocaine
Amphetamine
C
Vehicle
THC
D
AMPAR/NMDAR (+ 40 mV)
**
Vehicle
THC
E
Locked wheel
Free running
F
AMPAR/NMDAR (+ 40 mV)
1-h running distance (m)
1500
1000
500
0
*
Locked wheel
Free running
Free running
G
AMPAR/NMDAR (+ 40 mV)
r2 = 0.33; p = 0.11
600
800
1000
1200
1-h running distance (m)

Figure 1

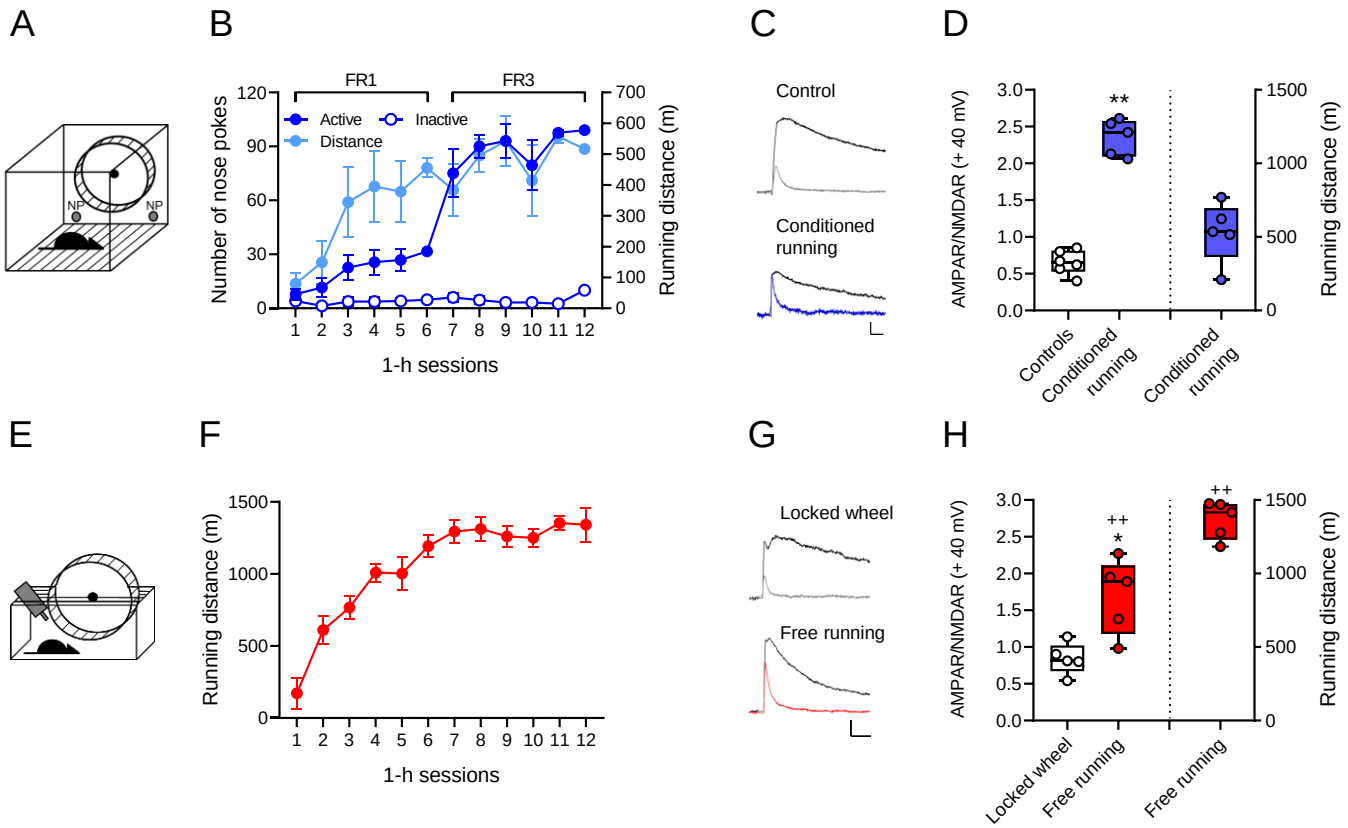
A
B
C
D
NP
NP
FR1
FR3
Active
Inactive
Distance
Number of nose pokes
Running distance (m)
1-h sessions
Control
Conditioned running
AMPAR/NMDAR (+ 40 mV)
**
Controls
Conditioned running
Conditioned running
E
F
G
H
Running distance (m)
1-h sessions
Locked wheel
Free running
AMPAR/NMDAR (+ 40 mV)
++
*
++
Locked wheel
Free running
Free running

Figure 2

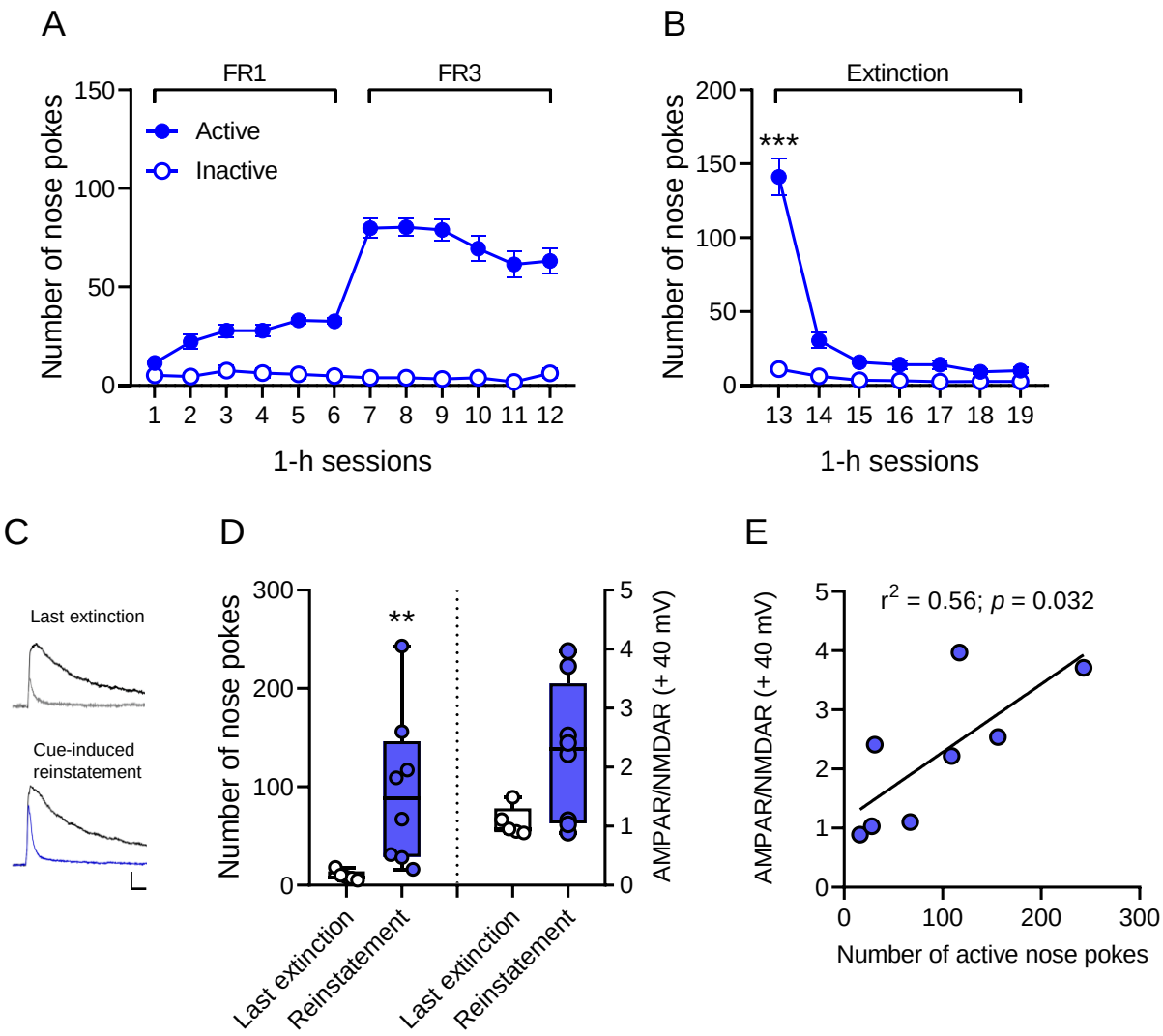
A
FR1
FR3
Active
Inactive
Number of nose pokes
150
100
50
0
1 2 3 4 5 6 7 8 9 10 11 12
1-h sessions
B
Extinction
***
Number of nose pokes
200
150
100
50
0
13 14 15 16 17 18 19
1-h sessions
C
Last extinction
Cue-induced reinstatement
D
Number of nose pokes
300
200
100
0
**
AMPAR/NMDAR (+ 40 mV)
5
4
3
2
1
0
Last extinction
Reinstatement
Last extinction
Reinstatement
E
r2 = 0.56; p = 0.032
AMPAR/NMDAR (+ 40 mV)
5
4
3
2
1
0
0
100
200
300
Number of active nose pokes

Figure 3

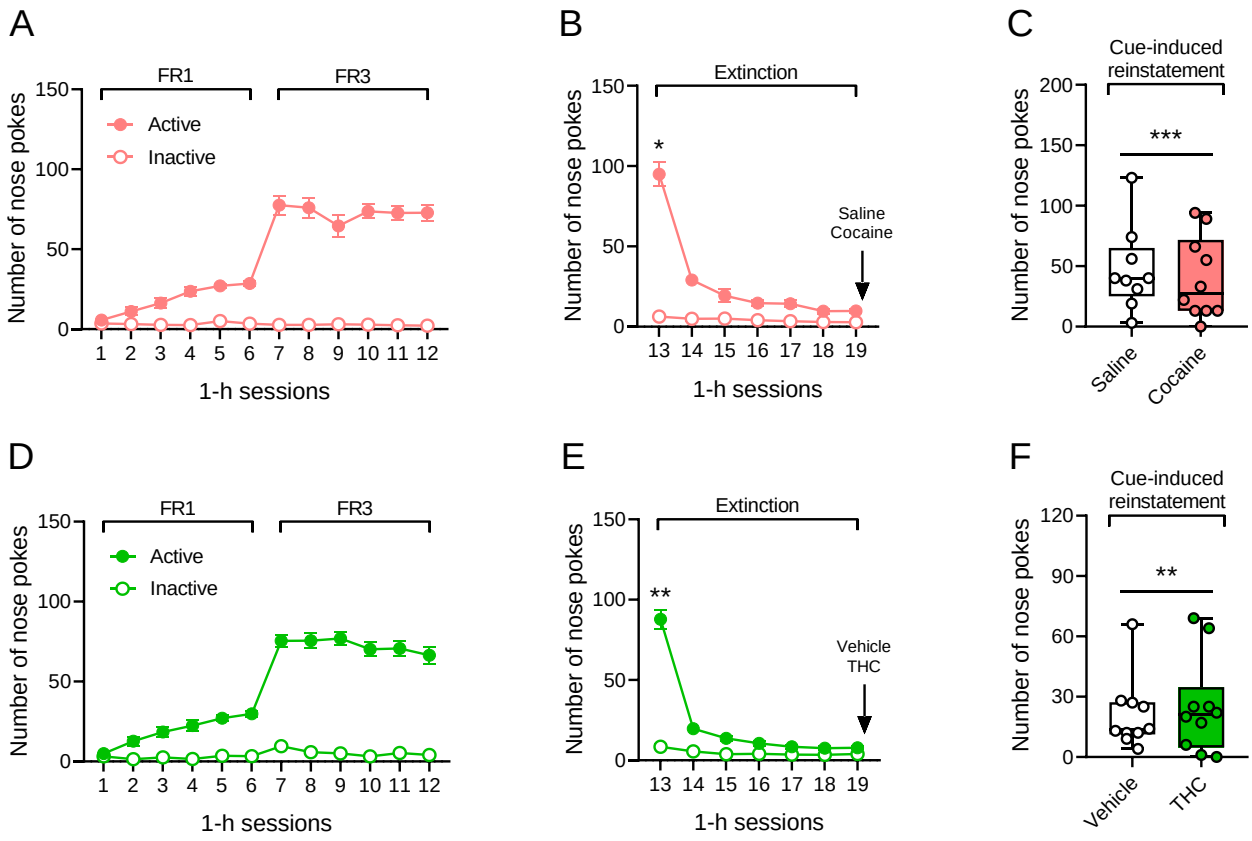
A
FR1
FR3
Active
Inactive
Number of nose pokes
1-h sessions
B
Extinction
Saline
Cocaine
C
Cue-induced reinstatement
***
Saline
Cocaine
D
E
Vehicle
THC
F
**

Figure 4