

1 **Title page**

2 **Wheel running during chronic nicotine exposure is protective against**
3 **mecamylamine-precipitated withdrawal and upregulates hippocampal $\alpha 7$**
4 **nACh receptors in mice**

5
6 **Short running title**

7 Exercise and nicotine withdrawal

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- 2 **References:** 74

1 Abstract

2 **Background and purpose.** Evidence suggests that exercise decreases nicotine withdrawal
3 symptoms in humans; however, the mechanisms mediating this effect are unclear. We
4 investigate, in a mouse model, the effect of exercise intensity during chronic nicotine exposure
5 on nicotine withdrawal severity, binding of $\alpha 4\beta 2^*$, $\alpha 7$ nicotinic acetylcholine (nAChR), μ -
6 opioid (μ receptors) and D_2 dopamine receptors, and on brain-derived neurotrophic factor
7 (BDNF) and plasma corticosterone levels.

8 **Experimental approach.** Male C57Bl/6J mice treated with nicotine (minipump, 24 mg kg⁻¹
9 day⁻¹) or saline for 14 days underwent one of three concurrent exercise regimes: 24, 2 or 0 hrs
10 day⁻¹ voluntary wheel running. Mecamylamine-precipitated withdrawal symptoms were
11 assessed on day 14. Quantitative autoradiography of $\alpha 4\beta 2^*$, $\alpha 7$ nAChRs, μ receptors and D_2
12 receptor binding was performed in brain sections of these mice. Plasma corticosterone and
13 brain BDNF levels were also measured.

14 **Key results.** Nicotine-treated mice undertaking 2 or 24 hrs day⁻¹ wheel running displayed a
15 significant reduction of withdrawal symptom severity compared with the sedentary group.
16 Wheel-running induced a significant upregulation of $\alpha 7$ nAChR binding in the CA2/3 area of
17 the hippocampus of nicotine-treated mice. Neither exercise nor nicotine treatment affected μ
18 or D_2 receptor binding or BDNF levels. Nicotine withdrawal increased plasma corticosterone
19 levels and $\alpha 4\beta 2^*$ nAChR binding, irrespective of exercise regimen.

20 **Conclusions and implications.** We demonstrate for the first time a profound effect of exercise
21 on $\alpha 7$ nAChRs of nicotine-dependent animals, irrespective of exercise intensity. These findings
22 shed light onto the mechanism underlining the protective effect of exercise in the development
23 of nicotine dependence.

24

25 **Abstract word count:** 250/250

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1 **Keywords:** Chronic nicotine, withdrawal, exercise, $\alpha 7$ nicotinic receptors, hippocampus

2

3 **Non-standard abbreviations:** BDNF (brain-derived neurotrophic factor); BLA (basolateral
4 amygdale); CA1 or 2/3 (regions of the hippocampus); CgCx (cingulate cortex); D₂ receptor
5 (dopamine D₂ receptor); DAMGO (D-Ala²-MePhe⁴-Gly-ol⁵ enkephalin); μ receptor (μ -opioid
6 receptor); nAChR (nicotinic acetylcholine receptor); NSB (non-specific binding); PAG
7 (periaqueductal grey); SEM (standard error of the mean); VTA (ventral tegmental area); ZT
8 (zeitgeber).

9

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1 Table of Links

Targets

GPCR^a

[μ receptor](#)

[D₂ receptor](#)

Ion Channels^b

[nicotinic acetylcholine receptor α7 subunit](#)

[nicotinic acetylcholine receptor α4 subunit](#)

[nicotinic acetylcholine receptor β2 subunit](#)

2

Ligands

[\[¹²⁵I\]α-bungarotoxin](#)

[\[³H\]raclopride](#)

[\[¹²⁵I\]epibatidine](#)

[\[³H\]DAMGO, D-Ala²-MePhe⁴-Gly-ol⁵ enkephalin](#)

3 These Table of Links list key protein targets and ligands in this article which are hyperlinked
4 to corresponding entries in [http:// www.guidetopharmacology.org](http://www.guidetopharmacology.org), the common portal for data
5 from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are
6 permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b}Alexander *et*
7 *al.*, 2013a; Alexander *et al.*, 2013b).

1 **Introduction**

2 More than fifty percent of attempts to quit smoking in the UK are not successful, which
3 is thought to be at least partly due to the limited efficacy of the substitution pharmacotherapies
4 currently available (The Health and Social Care Information Centre, 2012). Exercise, however,
5 has been shown to be of benefit as a non-pharmacological aid for treating nicotine dependence.
6 In particular, clinical and laboratory studies provide some evidence that exercise prior to
7 smoking cessation and/or during smoking cessation can reduce the severity of nicotine
8 withdrawal and craving following cessation of drug-taking and might be protective against
9 relapse (for reviews see Abrantes *et al.*, 2009; Haasova *et al.*, 2013; Taylor *et al.*, 2007b). With
10 regards to other drugs of abuse, *in vivo* animal studies showed that exercise can attenuate
11 priming- and cue-induced reinstatement of cocaine self-administration (Smith *et al.*, 2012;
12 Thanos *et al.*, 2013) and reduce morphine withdrawal symptoms (Balter & Dykstra, 2012;
13 Miladi-Gorji *et al.*, 2012), further supporting the beneficial effect of exercise in reducing drug
14 withdrawal symptoms and preventing relapse. Nonetheless, the frequency and intensity of
15 exercise needed, as well as the neurobiological mechanisms underpinning these beneficial
16 effects of exercise on reducing drug withdrawal and preventing relapse remain unclear.

17 Since neuronal nicotinic acetylcholine receptors (nAChRs) are the primary target of
18 nicotine (Barik & Wonnacott, 2009), the reinforcing compound in cigarettes (Picciotto &
19 Kenny, 2013), nAChRs are a central candidate system that may underlie the beneficial effect
20 of exercise in reducing nicotine withdrawal symptoms. Previous studies have shown that mice
21 lacking the $\alpha 4$ or $\beta 2$ subunits do not self-administer nicotine (Marubio *et al.*, 1999; Picciotto *et*
22 *al.*, 1998), while mecamylamine (nAChR antagonist)-precipitated withdrawal symptoms are
23 absent in $\beta 2$ and $\alpha 7$ knockout mice (Jackson *et al.*, 2008; Salas *et al.*, 2007), indicating $\alpha 4\beta 2^*$
24 and $\alpha 7$ nAChRs as essential mediators of nicotine dependence and withdrawal. However, the

1 effect of exercise on the nAChRs during chronic nicotine use and withdrawal has not yet been
2 studied.

3 The endogenous opioid system, and more specifically the μ -opioid system, has been
4 implicated in the effects of exercise (e.g. de Oliveira *et al.*, 2010), as well as during the different
5 phases of nicotine addiction/withdrawal (see le Merrer *et al.*, 2009). β -endorphin, an
6 endogenous opioid ligand for the μ -opioid receptor (μ receptor), is thought to mediate the
7 mood-enhancing effects of exercise via its actions on the the μ receptor (de Oliveira *et al.*,
8 2010), a concept referred to as “runner’s high”. With regards to nicotine addiction, nicotine
9 administration in mice lacking the μ receptor gene does not produce rewarding properties and
10 these mice have attenuated nicotine somatic withdrawal symptoms (Berrendero *et al.*, 2002).
11 Moreover, chronic nicotine administration results in higher expression of the μ receptor in the
12 ventral tegmental area of the brain in mice (Walters *et al.*, 2005) and naloxone, an opioid
13 receptor antagonist, triggers withdrawal symptoms in nicotine-dependent rats (Malin *et al.*,
14 1993) and in daily smokers (Krishnan-Sarin *et al.*, 1999). Although these findings clearly show
15 a key role of the μ receptor system in the mediation of both the mood-enhancing effects of
16 exercise and the addiction-related behavioural effects of nicotine administration and
17 withdrawal, it is not clear if μ receptors are involved in the beneficial effects of exercise on
18 nicotine dependence and abstinence. As a result, assessing if exercise in nicotine dependent
19 individuals affects the regulation of μ receptors in the brain will shed light into the mechanisms
20 underlining the beneficial effect of exercise on nicotine dependence and thus warrants further
21 investigation.

22 Nicotine withdrawal is associated with a reduction of dopaminergic tone in the striatum
23 (see Hadjiconstantinou *et al.*, 2011), and D₂ receptors are acutely downregulated during
24 nicotine withdrawal in rats (Scott *et al.*, 2007). Since there is clinical and pre-clinical evidence
25 to suggest that exercise may be able to counteract the hypofunction of the DAergic system by

1 specifically increasing brain D₂ receptor levels in different psychiatric conditions (Fisher *et*
2 *al.*, 2013; Vučković *et al.*, 2010), we postulated that exercise during drug exposure might be
3 exerting its beneficial effects against the development of nicotine dependence by upregulating
4 striatal D₂ receptors as well.

5 Another key mediator of drug addiction is brain-derived neurotrophic factor (BDNF).
6 For example, BDNF levels are elevated in the ventral tegmental area and nucleus accumbens
7 during withdrawal from chronic cocaine treatment (Tapia-Arancibia *et al.*, 2001) and in the
8 hippocampus following alcohol cessation in ethanol-dependent rats (Tapia-Arancibia *et al.*,
9 2001). Importantly, there is some evidence indicating that exercise decreases accumbal BDNF
10 expression (Strickland *et al.*, 2016), suggesting that exercise might be manifesting its beneficial
11 properties against nicotine dependence by reducing elevated BDNF levels.

12 This study aimed to investigate the effect of three different intensities of exercise during
13 chronic nicotine exposure on the development of physical dependence as measured by acute
14 mecamylamine-precipitated somatic withdrawal in mice, and to assess the expression of $\alpha 4\beta 2^*$
15 and $\alpha 7$ nAChRs, μ receptors, D₂ receptors and BDNF in the brains of these mice. As there is
16 some clinical evidence to suggest that exercise may be able to reduce nicotine withdrawal
17 symptoms by attenuating the reduction in cortisol levels observed in temporarily abstinent
18 smokers (Scerbo *et al.*, 2010), we also measured plasma corticosterone levels in these mice.

19

20

1 **Methods**

2 **Animal Welfare and Ethical Statement**

3 A total of 80 male C57Bl/6 mice (B&K Universal, UK) aged 8 weeks were individually
4 housed in Macrolom Type II Long cages fitted with a 13 cm diameter concentric free-turning
5 running wheel (ClockLab, Actimetrics, Wilmette, IL) in light-tight, sound-attenuated cabinets.
6 Mice were maintained in a 12:12 hr light/dark cycle in ~~a-reverse~~an altered phase light protocol
7 (lights off 11:00 AM). Animals had *ad libitum* access to food and water throughout the
8 experiment. Animal work procedures were carried out in accordance with the Animal
9 (Scientific Procedures) Act 1986 Amendment Regulations (SI 2012/3039) under the project
10 licence PPL 70/7203, approved on 17th February 2011, and reported according to ARRIVE
11 guidelines. A mouse model was used in this study as it is commonly used to assess the
12 neurobiological mechanisms underpinning nicotine addiction and exercise. The exact group
13 size for each treatment/exercise group is provided for each experiment in Table 1. The
14 experimenter who performed the minipump surgeries and injected the animals was aware of
15 the pharmacological treatments and exercise regimen. Running wheel responses were
16 registered by an automated software, and the analysis of the behavioural and biochemical
17 autoradiographic binding outcomes of the study were carried out by researchers who were
18 blinded to the experimental/treatment groups. No animals were excluded from the analysis.
19 However, three animals died following minipump implantation.

20 **Assessment of Running Wheel Activity**

21 Mice were randomly assigned to one of three running wheel conditions and treated with
22 either nicotine or saline: wheels unlocked 24 hrs day⁻¹ (n=13); wheels unlocked 2 hrs day⁻¹
23 (n=12–14); and wheels unlocked 0 hr day⁻¹ (sedentary group, n=12–13). In the 2 hrs day⁻¹ group
24 wheels were unlocked at 13–15 zeitgeber time (ZT), as this is the peak activity time for
25 C57Bl/6J male mice on a 12:12 hr light/dark cycle (Hasan *et al.*, 2011). To determine profiles

1 of average running wheel activity, the total number of wheel revolutions day⁻¹ was converted
2 into distance run for the 7 days of habituation and 14 days of treatment (nicotine or saline
3 delivered via minipumps).

4 **Minipump preparation and implantation**

5 After habituating the mice in their running wheel condition for 7 days (see above), mice
6 were treated with a chronic, 14-day, nicotine administration regimen, as previously described
7 (Zanos *et al.*, 2015), with minor modifications. Briefly, mice were surgically implanted with
8 subcutaneous osmotic minipumps (Model 2002, Alzet®, Cupertino, CA) containing saline or
9 (-)-nicotine hydrogen tartrate (24 mg kg⁻¹ day⁻¹; Sigma Aldrich, Poole, UK) in sterile saline
10 delivering a constant flow at a rate of 0.5 µl hour⁻¹ for a period of 14 days. All nicotine
11 concentrations are expressed as nicotine free base. Drug dose was selected to achieve blood
12 nicotine levels comparable to the physiologically-relevant concentrations measured in plasma
13 of human smokers (see Matta *et al.*, 2007). For minipump implantation, animals were
14 anaesthetised with a volatile isoflurane anaesthetic (4.0 %) (Isoflo, Abbott Laboratories Ltd.,
15 Kent, UK), which was vaporised in 95 % O₂ / 5 % CO₂ gas and delivered by a U400 anaesthetic
16 unit (Univentor, Royem Scientific, Luton, UK) at a flow rate of approximately 450 ml min⁻¹
17 isoflurane/oxygen vapor mixture (3.5%–4.5%; Isoflo, Abbott Laboratories Ltd, Maidenhead,
18 Berkshire, UK). The animals were placed in the anaesthetic chamber for 1 min until the righting
19 reflex was lost and were subsequently placed under a mask delivering anaesthesia throughout
20 the surgery. Mice were injected with a non-opioid analgesic (Metacam, 1.5 mg kg⁻¹, s.c.). A
21 single incision along the midline of the back of each animal was made and osmotic mini-pumps
22 were placed in parallel position to the spine. The flow operator was pointing away from the
23 incision site. The incision was closed using 2–3 Michele clips (11 x 2.5 mm). Upon completion
24 of the surgical procedure mice were allowed to recover in heated-recovery chambers until their
25 righting reflex returned and were then placed back into their home cages.

1 **Assessment of nicotine withdrawal severity**

2 Fourteen days after minipump implantation all animals were injected with
3 mecamylamine (3 mg kg⁻¹, subcutaneously (s.c.); Sigma Aldrich, Poole, UK) (Damaj *et al.*,
4 2003) and immediately assessed for nicotine somatic withdrawal symptoms. Mice were
5 videotaped and observed for 30 mins in clear plastic activity cages for somatic withdrawal
6 symptoms, according to the scale developed by Castañé *et al.* (2002). The following abstinence
7 signs were evaluated during a 30-min period after mecamylamine injection: body tremor,
8 ptosis, wet dog shakes, rearing, teeth chattering, paw tremor, scratching, genital licks, sniffing
9 and piloerection. A global withdrawal score was calculated for each animal by giving each
10 individual symptom a relative weight: 0.5 for each episode of wet dog shake, front paw tremor,
11 sniffing, rearing and scratching; and 1 for appearance or 0 for non-appearance within each 5-
12 min bin for the presence of ptosis, genital licks, tremor, piloerection and teeth chattering. A
13 composite of all these individual withdrawal symptoms was calculated to make up a global
14 withdrawal symptom score. Scoring of behaviour was carried out by two independent observers
15 blind to the treatment protocol.

16 Thirty mins after the end of withdrawal assessment mice were euthanised with a 20-sec
17 CO₂ exposure, and trunk blood was collected, following decapitation, in EDTA-containing
18 eppendorf tubes. Brains were excised and immediately frozen in isopentane ~~solution~~ (-20 °C)
19 and then stored at -80 °C for autoradiography or BDNF measurements. Trunk blood was
20 centrifuged (240 x g at 4 °C for 15 min) and the plasma stored at -20 °C for subsequent analysis
21 of corticosterone content.

22 **Quantitative receptor autoradiography**

23 Brains from some animals used for the behavioural studies (for exact number, see Table
24 1) were sectioned in a cryostat (Zeiss Hyrax C 25, Carl Zeiss AG, Oberkochen, Germany), at
25 -21 °C. 20 µm coronal sections were cut at 300 µm intervals, from rostral to caudal levels, and

1 thaw-mounted onto gelatine coated ice-cold microscope slides and processed for
2 autoradiography. Adjacent sections were cut for determination of total and non-specific (NSB)
3 binding. Sections were stored at -20 °C prior to radioligand binding.

4 Quantitative autoradiography was performed on brain sections for $\alpha 4\beta 2^*$, $\alpha 7$, μ
5 receptors and D₂ receptors using [¹²⁵I]epibatidine (100 pM ± 20 nM cytisine), [¹²⁵I] α -
6 bungarotoxin (α -Bgtx; 3 nM), [³H]D-Ala²-MePhe⁴-Gly-ol⁵ enkephalin (DAMGO; 4 nM) and
7 [³H]raclopride (4 nM), respectively, according to established protocols (Georgiou *et al.*, 2016;
8 Metaxas *et al.*, 2013; Wright *et al.*, 2016), with minor modifications (see Supplemental
9 Information).

10 **Plasma corticosterone and brain BDNF measurements**

11 *Plasma corticosterone levels:* Plasma samples from trunk blood were assayed for
12 corticosterone content using a rat/mouse [¹²⁵I]-corticosterone radioimmunoassay kit (MP
13 Biomedicals, New York, NY), according to manufacturer's instructions.

14 *Brain BDNF levels:* Brains from some animals used for behavioural studies (for exact
15 number, see Table 1) were defrosted in distilled water and the frontal cortex, striatum (i.e.,
16 nucleus accumbens and caudate putamen) and hippocampus dissected and weighed. The key
17 role of BDNF in these brain regions has been extensively demonstrated in the drug addiction
18 field (Li & Wolf, 2015). These brain regions were selected based on previous evidence for
19 alterations of BDNF following chronic drug use (see McGinty *et al.*, 2010). Each sample was
20 homogenised by ultrasonification in lysis buffer containing 100 mM PIPES, 500 mM NaCl, 15
21 mM NaN₃, 20% BSA, 2.5 mM EDTA, 0.2 % TRITON X-100 and EDTA-free protease
22 inhibitor cocktail (P8340, Sigma Aldrich, Poole, UK), pH 7 at room temperature. Total BDNF
23 protein levels in homogenates were determined using the Promega BDNF E_{max}[®] ImmunoAssay
24 System with acid treatment according to manufacturer's instructions (Promega, Madison, WI).

25 **Data analysis and statistical procedures**

1 All data are presented as mean \pm SEM and were analysed using Statistica (STATsoft,
2 Inc., version 10, Tulsa, OK). ANOVAs were followed by Bonferroni *post-hoc* tests where
3 significance was achieved ($p < 0.05$). Withdrawal data were analysed using non-parametric tests
4 followed by *post-hoc* tests where significance was $p < 0.05$. For details on statistical analyses
5 see *Supplemental Information*. ANOVA results and precise sample sizes are detailed in Table
6 1. All the data and statistical analyses comply with the recommendations on experimental
7 design and analysis in pharmacology (Curtis *et al.*, 2015).

8 **Materials**

9 (-)-Nicotine hydrogen tartrate, mecamylamine, cytisine and sulpiride were purchased
10 from Sigma Aldrich, Poole, UK. BDNF kits and corticosterone kits were purchased from
11 Promega, Madison, WI, and MP Biomedicals, New York, NY, respectively. [¹²⁵I]epibatidine
12 (specific activity 2200 Ci mmol⁻¹), [¹²⁵I] α -Bungarotoxin (specific activity 108.8 Ci mmol⁻¹),
13 [³H]DAMGO (specific activity 51.5 Ci mmol⁻¹) and [³H]raclopride (specific activity 60 Ci
14 mmol⁻¹) used for autoradiographic binding experiments were purchased from PerkinElmer,
15 Waltham, MA.

1 **Results**

2 **Activity profiles of saline- and nicotine-treated mice**

3 As expected, no wheel-running activity was recorded for the 0 hrs day⁻¹ wheel-running
4 group. Total activity per day was determined for animals in the 2 and 24 hrs day⁻¹ wheel-
5 running groups throughout the habituation and treatment periods in order to assess whether
6 mice reached a steady-state of activity (Fig. 1A). Three-way repeated measures ANOVA
7 revealed a significant effect of exercise; the 24 hrs day⁻¹ group showed higher activity
8 throughout the habituation and treatment phases of the experiment. There was no significant
9 effect of nicotine treatment on wheel-running activity (Fig. 1A; see Table 1).

10 **Effect of different exercise regimes on severity of nicotine withdrawal syndrome**

11 Individual withdrawal symptoms were analysed and a composite total withdrawal
12 factor was calculated (Fig. 1B). Non-parametric Kruskal-Wallis test revealed a significant
13 effect of exercise on withdrawal in nicotine-treated mice. Multiple Mann-Whitney *U*-tests
14 showed that precipitated withdrawal induced significantly higher withdrawal symptoms in
15 nicotine-treated mice in the 0 hrs day⁻¹ group only compared with the saline-treated controls
16 ($U=27.50$, $z=-2.75$, $p=0.003$, 1-tailed), but showed no difference between saline- and nicotine-
17 treated mice within the 2 or 24 hrs day⁻¹ groups. Moreover, mecamylamine administration
18 induced higher severity of withdrawal symptoms in nicotine-treated mice in the sedentary
19 group compared with nicotine-treated mice in the 2 or 24 hrs day⁻¹ wheel access groups
20 ($U=26.50$, $z=2.63$, $p=0.004$ and $U=32.00$, $z=2.50$, $p=0.006$, 1-tailed, respectively; Dunn's
21 corrected α -level=0.025). There was also no difference in severity of withdrawal between
22 nicotine-treated mice in 2 and 24 hrs day⁻¹ wheel access groups (Fig. 1B; see Table 1).
23 Interestingly, when different components of the withdrawal symptoms were analysed,
24 mecamylamine-precipitated withdrawal in nicotine treated sedentary animals induced an

1 increase of paw tremors, sniffing and rearing which was absent in the groups exposed to
2 exercise regimes (Supplementary Fig. S1).

3 **Effect of exercise on $\alpha 4\beta 2^*$ nAChR binding in nicotine-treated mice**

4 Levels of $\alpha 4\beta 2^*$ nAChR binding were determined using cytisine-sensitive
5 [125 I]epibatidine binding in brain regions of mecamylamine-precipitated saline- or nicotine-
6 treated mice with 0, 2 and 24 hrs day⁻¹ running wheel access (Fig. 2A,B; Supplementary Table
7 S1). Cytisine-resistant binding was only present in the medial habenula (MHb) for all groups
8 (Fig. S2), indicating a high level of non- $\alpha 4\beta 2^*$ (most likely $\alpha 3\beta 4^*$) heteromeric nAChR
9 binding in that region. A two-way ANOVA found no significant nicotine ($p > 0.05$) or exercise
10 ($p > 0.05$) effects within that region (Table 1). Two-way ANOVA followed by Bonferroni *post-*
11 *hoc* in each region revealed significant, nicotine-induced upregulation of cytisine-sensitive
12 [125 I]epibatidine binding in the frontal association, as well as the prelimbic cortex, motor
13 cortex, cingulate cortex, nucleus accumbens core and shell, hypothalamus, substantia nigra
14 pars compacta and ventral tegmental area irrespective of exercise regimen (Fig. 2B). $\alpha 4\beta 2^*$
15 nAChR binding was also upregulated in the motor, somatosensory, piriform, retrosplenial and
16 auditory cortices, as well as the medial septum, ventral limb of the diagonal band of Broca,
17 olfactory tubercle and subiculum of nicotine-treated animals compared with saline controls
18 irrespective of exercise regimen (Supplementary Table S1). No significant treatment effect was
19 observed in the nucleus accumbens core, thalamus or the hippocampus. There were no exercise
20 or interaction effects in any of the brain regions analysed (see Table 1 and Supplementary Table
21 S1).

22 **Effect of exercise on $\alpha 7$ nAChR binding in nicotine-treated mice**

23 $\alpha 7$ nAChR density was determined by [125 I] α -bungarotoxin binding in the brain of
24 mecamylamine-precipitated saline- or nicotine-treated mice that were permitted 0, 2 and 24 hrs
25 day⁻¹ running wheel access (Fig. 3A,B; Supplementary Table S2). Two-way ANOVA in each

1 brain region revealed a significant treatment effect in the cingulate cortex, endopiriform
2 nucleus, motor cortex, clostrum, CA1 region of the hippocampus, amygdala and hypothalamus
3 (Fig. 3B). In the motor cortex, where a significant ANOVA interaction between treatment and
4 exercise was identified (see Table 1), we demonstrated a significant decrease in $\alpha 7$ binding in
5 the 24 hrs day⁻¹ exercise saline-treated group compared with their sedentary controls ($p < 0.05$),
6 which was absent in nicotine-treated animals (Fig. 3B). Moreover, saline-treated mice that were
7 permitted 24-hour day⁻¹ running-wheel access showed a significantly lower $\alpha 7$ binding
8 compared to nicotine-treated mice which were permitted the same exercise schedule (Fig. 3B).

9 Two-way ANOVA revealed a significant exercise effect ($p < 0.05$), a significant
10 treatment effect ($p < 0.05$) and a significant exercise x treatment interaction effect ($p < 0.05$) in
11 the CA2/3, clearly demonstrating an interaction effect of nicotine and exercise on $\alpha 7$ nAChR
12 upregulation in the CA2/3. Nicotine treatment elicited higher levels of $\alpha 7$ binding in the CA2/3
13 hippocampal area of mice exposed to 2 or 24 hrs day⁻¹ running wheel access ($p < 0.05$),
14 compared to nicotine-treated sedentary animals and compared to their saline, exercise-
15 matching controls. Mice with 24 hrs day⁻¹ access to a running-wheel also displayed higher
16 levels of $\alpha 7$ nAChR binding in the CA2/3 of saline-treated mice compared with mice in the 0
17 hrs day⁻¹ saline-treated group (Fig. 3B; Table 1).

18 **Effect of exercise on μ receptor binding in nicotine-treated mice**

19 Binding of the μ receptor was determined by [³H]DAMGO binding in brain regions of
20 mecamylamine-precipitated saline- or nicotine-treated mice permitted 0, 2 and 24 hrs day⁻¹
21 running wheel access (Fig. 4A). Two-way ANOVA for each brain region did not reveal any
22 effect of treatment or exercise, nor interactions between these factors (Fig. 4A; Table 1).

23 **Effect of exercise on D₂ receptor binding in nicotine-treated mice**

24 Binding of D₂ receptors was determined by [³H]raclopride binding in brain regions of
25 mecamylamine-precipitated saline- or nicotine-treated mice permitted 0, 2 and 24 hrs day⁻¹

1 running wheel access (Fig. 4B). Two-way ANOVA for each brain region revealed no
2 significant changes in [³H]raclopride binding in any of the regions analysed (Fig. 4B; Table 1).

3 **Effect of exercise on brain BDNF in nicotine-treated mice**

4 The level of free BDNF in the prefrontal cortex, striatum and hippocampus of
5 mecamylamine-precipitated saline- or nicotine-treated permitted 0, 2 and 24 hrs day⁻¹ running
6 wheel access was determined using an ELISA. Two-way ANOVA for each brain region
7 showed no significant changes in any of the regions analysed (Fig. 5A; Table 1).

8 **Effect of exercise on plasma corticosterone in nicotine-treated mice**

9 Plasma corticosterone levels were determined by radioimmunoassay of
10 mecamylamine-precipitated saline- or nicotine-treated mice permitted 0, 2 and 24 hrs day⁻¹
11 running wheel access (Fig. 5B). Two-way ANOVA revealed a significant increase of plasma
12 corticosterone levels induced by nicotine treatment (treatment effect; Table 1) irrespective of
13 exercise regimen. No effects of exercise were found.

14

1 **Discussion**

2 The present study highlights the beneficial effect of exercise during nicotine exposure
3 in markedly reducing the severity of nicotine somatic withdrawal symptoms, an effect that is
4 accompanied by an upregulation of the hippocampal $\alpha 7$ nAChRs. These findings support the
5 protective effect of exercise preceding smoking cessation against the development of physical
6 dependence, which may aid smoking cessation by reducing withdrawal symptom severity.
7 Moreover, we propose a novel mechanism of action of exercise involving hippocampal $\alpha 7$
8 nAChRs.

9 Two hrs day⁻¹ access to a running wheel was equally effective in attenuating nicotine
10 withdrawal symptoms as continuous 24 hrs day⁻¹ access. This is consistent with human clinical
11 studies showing that just 10 mins of moderate intensity exercise during smoking cessation is
12 sufficient to reduce cigarette craving, withdrawal symptoms and cue-induced cravings (Scerbo
13 *et al.*, 2010; Taylor *et al.*, 2007a; Ussher *et al.*, 2001), supporting the translational validity of
14 our mouse model. In rodent models, 2 hrs day⁻¹ access to running wheels during a period of
15 abstinence from nicotine self-administration decreased subsequent nicotine-seeking in rats
16 (Sanchez *et al.*, 2013), demonstrating a beneficial effect of exercise on nicotine craving during
17 abstinence. However, 2-hr daily exercise failed to prevent cue-induced reinstatement of
18 nicotine-seeking (Sanchez *et al.*, 2013). This effect does not preclude the possibility that a more
19 intense exercise schedule could have prevented reinstatement of nicotine-seeking after
20 extinction; however, this hypothesis needs to be investigated further. Here, we show that
21 exercise exposure concurrent with nicotine administration is able to significantly reduce
22 physical symptoms of withdrawal, which might underlie its ability to reduce nicotine craving
23 during abstinence. It is important to note that, based on our results, it is not possible to ascertain
24 if exercise during the withdrawal phase (irrespective of exercise during the nicotine exposure
25 phase) would be sufficient to decrease withdrawal severity, as mice were not exposed to an

1 exercise regime during the withdrawal phase. Studies assessing the effects of exercise during
2 un-precipitated nicotine withdrawal are warranted to address this question. Nonetheless, the
3 data clearly suggest that exercise preceding smoking cessation might be able to increase the
4 chances of abstinence from smoking by reducing acute physical withdrawal symptom severity.
5 We also aimed to identify possible neurobiological mechanisms underlying this effect.

6 $\alpha 4\beta 2^*$ nAChR upregulation was observed in most brain regions of mice exposed to
7 chronic nicotine administration followed by mecamylamine-precipitated withdrawal,
8 irrespective of exercise regimen, demonstrating that exercise does not influence nicotine-
9 induced $\alpha 4\beta 2^*$ nAChR upregulation. Upregulation of $\alpha 4\beta 2^*$ nAChR following prolonged
10 exposure to nicotine has been consistently shown in cigarette smokers ~~(see Fowler *et al.*,~~
11 ~~2008)~~(Breese *et al.*, 1997; Cosgrove *et al.*, 2009) and animal models of nicotine administration
12 (e.g. Metaxas *et al.*, 2013), and was associated with increased self-administration of the drug
13 (Hambsch *et al.*, 2014). The upregulation is almost certainly due to chronic nicotine treatment
14 not ‘mecamylamine-precipitated withdrawal’ indicating that this $\alpha 4\beta 2^*$ nAChR upregulation
15 persists at least following acute precipitated withdrawal. The present results demonstrate that
16 exercise does not influence nicotine-induced $\alpha 4\beta 2^*$ nAChR upregulation and thus is unlikely
17 to be involved in the mechanism underlying the beneficial effect of exercise during nicotine
18 exposure on nicotine withdrawal symptoms.

19 Moreover, we showed that $\alpha 7$ nAChRs are almost globally upregulated in most of the
20 brain regions analysed in chronically nicotine-treated mice undergoing mecamylamine-
21 precipitated withdrawal compared with saline-treated controls. This finding is in line with
22 previous studies showing that $\alpha 7$ nAChRs are upregulated in response to chronic nicotine
23 exposure (Metaxas *et al.*, 2013), indicating that this upregulation persists during acute
24 precipitated withdrawal. Importantly, we demonstrated that hippocampal $\alpha 7$ nAChR binding
25 is regulated by exercise since 2 or 24 hrs day⁻¹, but not 0 hrs day⁻¹ running wheel access induced

1 a significant upregulation of $\alpha 7$ nAChR binding in the CA2/3 region of the hippocampus
2 irrespective of nicotine/saline treatment schedule, suggesting the presence of a specific
3 exercise-induced effect on $\alpha 7$ nAChRs.

4 While exercise increases $\alpha 7$ nAChRs binding in saline treated and nicotine treated
5 animals, the upregulation in the exercise plus nicotine group was found to be significantly
6 higher than the saline plus exercise group, indicating an exercise x nicotine interaction on $\alpha 7$
7 nAChR upregulation in the CA2/3 of the hippocampus. This upregulation is concomitant with
8 the complete abolition of somatic nicotine withdrawal symptoms in chronically nicotine treated
9 mice exposed to exercise. Although, on the basis of the present data alone it would be ~~wrong~~
10 ~~presumptuous~~ to assume any causal relationship between the protective effect of exercise on
11 somatic withdrawal symptoms and $\alpha 7$ nAChR hippocampal upregulation, there is considerable
12 evidence linking $\alpha 7$ nAChRs and with at least some of the somatic symptoms of mecamylamine
13 induced nicotine withdrawal. Salas *et al.* (2007) reported a decrease of shaking and scratching
14 but not wet dog shakes and head nods in nicotine treated $\alpha 7$ knockout mice undergoing
15 mecamylamine-precipitated withdrawal. Interestingly, we also show in the present study an
16 abolition of mecamylamine-induced withdrawal paw shakes in nicotine treated mice exposed
17 to exercise, an effect which was concomitant to a hippocampal CA2/3 $\alpha 7$ nAChR upregulation,
18 suggesting that there may be a link between $\alpha 7$ nAChR upregulation and the protective effect
19 of exercise on nicotine withdrawal symptoms. Moreover, the selective $\alpha 7$ nAChR agonist
20 PNU282987 and the high $\alpha 7$ /low $\alpha 4\beta 2^*$ efficacy agonist varenicline (Chantix, New York, NY)
21 have shown good efficacy in decreasing motivation to consume nicotine (Brunzell *et al.*, 2010;
22 Harmey *et al.*, 2012) and in reducing withdrawal symptoms and craving (Rankin & Jones,
23 2011). It is important to note that $\alpha 7$ nAChRs have also been implicated in ~~nicotine withdrawal-~~
24 ~~associated the anhedonia/affective disruptive effect associated with nicotine withdrawal~~
25 (Stoker *et al.*, 2012), which is ~~entirely~~ clinically relevant ~~effects~~ as it constitutes a ~~potential~~

1 motivational trigger to relapse. Even more intriguingly, recent data points specifically to the
2 hippocampal $\alpha 7$ nAChRs as key modulators of negative affect (Mineur *et al.*, 2017) which
3 makes our hypothesis for a direct link between the protective effect of exercise on the negative
4 consequences of nicotine abstinence and $\alpha 7$ hippocampal upregulation even more appealing. It
5 is of course impossible to know based on the current study whether those upregulated receptors
6 are desensitized or active and if these lead to downstream adaptations that may protect the
7 development of physical dependence. Future studies should focus on the biological
8 significance of this upregulation in order to test this hypothesis.

9 Interestingly, although $\alpha 4\beta 2^*$ nAChRs have been recognised to play a key role in the
10 cognitive impairment associated with nicotine withdrawal (Simmons & Gould, 2014), $\alpha 7$
11 nAChR activation has been shown to improve cognition, which is impaired during nicotine
12 withdrawal in both mice and humans (Dajas-Bailador *et al.*, 2004; Parrott *et al.*, 1996;
13 Wilkinson *et al.*, 2013). Administration of varenicline, a partial $\alpha 4\beta 2^*$ agonist and a full
14 $\alpha 7/\alpha 3\beta 4$ nAChR agonist, has been found to attenuate contextual fear conditioning during
15 nicotine withdrawal (Raybuck *et al.*, 2008). In addition, upregulation of $\alpha 7$ nAChRs has been
16 associated with the pro-cognitive effects of $\alpha 7$ agonists (Christensen *et al.*, 2010). Therefore,
17 given the key role of the hippocampus as a brain region involved in nicotine withdrawal
18 mechanisms related to cognitive effects, future studies are warranted to directly investigate
19 whether exercise exerts its beneficial effect in attenuating the cognitive deficits induced by
20 nicotine withdrawal via an enhancement of hippocampal $\alpha 7$ nAChRs. It is important to note
21 that many other nAChR subtypes have been implicated in somatic symptoms of withdrawal
22 including $\alpha 3$, $\alpha 5$, $\alpha 4$ and $\alpha 2$ nAChR subtypes (see review by Jackson *et al.*, 2015). Of particular
23 interest is the emergence of the habenula-interpenduncular nucleus and cytosine resistant
24 $\alpha 3\beta 4^*$ nAChRs in the manifestation of somatic withdrawal symptoms (Salas *et al.*, 2009;
25 Baldwin *et al.*, 2011). Nonetheless, no nicotine nor exercise effect was observed in habenular

1 cytosine resistant [¹²⁵I]epibatidine binding sites which most likely represent $\alpha 3\beta 4^*$ nAChRs
2 (Fig. S2). This finding plausibly suggests that exercise is unlikely to affect $\alpha 3\beta 4^*$ nAChR
3 density in the habenula and thus may not play a key role in the protective effect of exercise on
4 nicotine dependence. Future research should be directed in the investigation of $\alpha 3$ and $\alpha 5$ in
5 the effect of exercise in decreasing nicotine withdrawal symptoms.

6 BDNF, which has been shown to be increased in the hippocampus following exercise
7 (Fuss *et al.*, 2010) and chronic nicotine treatment (Aydin et al 2012; Czubak 2009; Kenny
8 2009), has also been shown to specifically upregulate the intracellular pool of $\alpha 7^-$, but not $\beta 2^-$ -
9 , containing nAChRs in cultured hippocampal neurons (Massey *et al.*, 2006; Zhou *et al.*, 2004).
10 As a result, we postulated that the observed exercise-induced region-specific upregulation of
11 $\alpha 7$ nAChR in the brain of nicotine-treated mice might be mediated by an elevation of BDNF
12 levels. However, in the present study, neither voluntary wheel-running nor nicotine treatment
13 had any effect on BDNF levels in the hippocampus, striatum or prefrontal cortex. This
14 discrepancy with the literature may be due to different species, exercise regimens, treatment
15 period and nicotine doses tested. For instance, while nicotine downregulates BDNF in the
16 short-term (2–24 hrs), there is a positive correlation between the amount of exercise and BDNF
17 production (for review, see Erickson *et al.*, 2011). The species differences between the
18 published studies and our findings might also explain these discrepancies as environmental
19 enrichment, including use of running wheels, increased hippocampal BDNF in rats (Ickes *et*
20 *al.*, 2000), but not in mice (Rueda *et al.*, 2012). Nonetheless, our data do not support the
21 hypothesis that exercise during nicotine exposure might affect nicotine withdrawal symptoms
22 via a mechanism involving hippocampal, striatal or cortical BDNF upregulation, nor that
23 changes in BDNF levels in these brain regions are involved in the observed exercise-induced
24 upregulation of hippocampal $\alpha 7$ nAChRs following nicotine treatment.

1 Exercise has previously been shown to upregulate D₂ receptors in humans (Fisher *et*
2 *al.*, 2013) and rodents (Vučković *et al.*, 2010), however the present study found no change in
3 D₂ receptor binding following either exercise or nicotine withdrawal. The reason behind this
4 discrepancy may lie in the fact that exercise-induced D₂ receptor upregulation was previously
5 observed in a mouse model of Parkinsons' disease (Vučković *et al.*, 2010), indicating that
6 exercise-induced upregulation only occurs in compensation for loss of DAergic tone; this loss
7 does not appear to happen in our mouse model of precipitated nicotine withdrawal.
8 Nevertheless, our findings do not preclude the possibility that changes in the downstream D₂
9 receptor signalling pathway, or functional changes at the receptor, might be involved in the
10 mechanism underpinning the effects of exercise during nicotine exposure on acute somatic
11 withdrawal symptoms and warrants further investigation.

12 In contrast to $\alpha 7$ nAChRs, no exercise or nicotine treatment interaction effects were
13 observed in μ receptor binding in any of the regions analysed, suggesting that changes in this
14 receptor system is unlikely to be part of the mechanism underpinning the beneficial effect of
15 exercise during nicotine exposure on reducing nicotine withdrawal symptoms. This is
16 somewhat surprising considering the plethora of evidence demonstrating a key role of the
17 endogenous opioid system in the mechanism underlying the rewarding effect of exercise and
18 nicotine (de Oliveira *et al.*, 2010; Berrendero *et al.*, 2002). Nonetheless, the findings from our
19 study clearly suggest that any involvement of the opioid system is likely to be at the receptor
20 signalling and/or the opioid peptide level rather than at the receptor expression level.

21 Although exercise has been suggested to influence nicotine withdrawal and craving via
22 a possible modulation of the hypothalamic-pituitary adrenal axis activity (Scerbo *et al.*, 2010),
23 here we show that exercise during nicotine exposure had no effect on corticosterone levels in
24 saline- or nicotine-treated mice, indicating that its protective effects on nicotine dependence
25 are not mediated by its actions on the hypothalamic-pituitary adrenal axis level. However,

1 consistent with the high levels of plasma cortisol observed in regular smokers (al'Absi *et al.*,
2 2003; Field *et al.*, 1994), we found an elevation of corticosterone levels in mecamylamine-
3 precipitated nicotine-withdrawn mice irrespective of exercise regimen, supporting the
4 translational validity of our mouse model of chronic nicotine administration.

5 Other than the opioid, dopamine, nicotinic system, all investigated in this study, the
6 endocannabinoid system may also play a key role in the mechanism underlining the effect of
7 exercise in reducing nicotine withdrawal symptoms. Stimulation of the endogenous
8 cannabinoid type-1 (CB₁) receptors is a prerequisite for voluntary running in mice (Dubreucg
9 *et al.*, 2013) and enhancement of two endogenous endocannabinoids (anandamide and 2-
10 arachidonylglycerol), by inhibition of their metabolic enzyme FAAH was shown to reduce
11 nicotine withdrawal symptoms in rats (Cippitelle *et al.*, 2011). Investigation of the role of the
12 endocannabinoid system in the beneficial effect of exercise in nicotine dependence would be
13 an important an interesting concept for future investigation.

14 In conclusion, our results demonstrate the effectiveness of even a moderate amount of
15 exercise during nicotine exposure in attenuating nicotine withdrawal symptoms and point
16 toward the hippocampal $\alpha 7$ nAChR system as a potential mechanism underlining this effect.
17 These findings may also have implications for the development of targeted interventions prior
18 to smoking cessation which may increase the chances of smoking cessation.

19

20

1 **Author contributions**

2 H. K., A.B., M. C., Y. C. and I. K. conceived and designed the experiments. H. K., and A.V.
3 R. performed the experiments. H. K., P.G., P.Z. and A. B. analysed the data. A. V. R. and R.
4 C. contributed reagents/materials/analysis tools. H. K., A.B., M. C., P.G. and P.Z. wrote the
5 manuscript.

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11 **Conflict of Interest**

12 The authors declare no conflict of interest.

13 **Declaration of transparency and scientific rigour**

14 This Declaration acknowledges that this paper adheres to the principles for transparent
15 reporting and scientific rigour of preclinical research recommended by funding agencies,
16 publishers and other organisations engaged with supporting research.

17

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Figures and figure legends

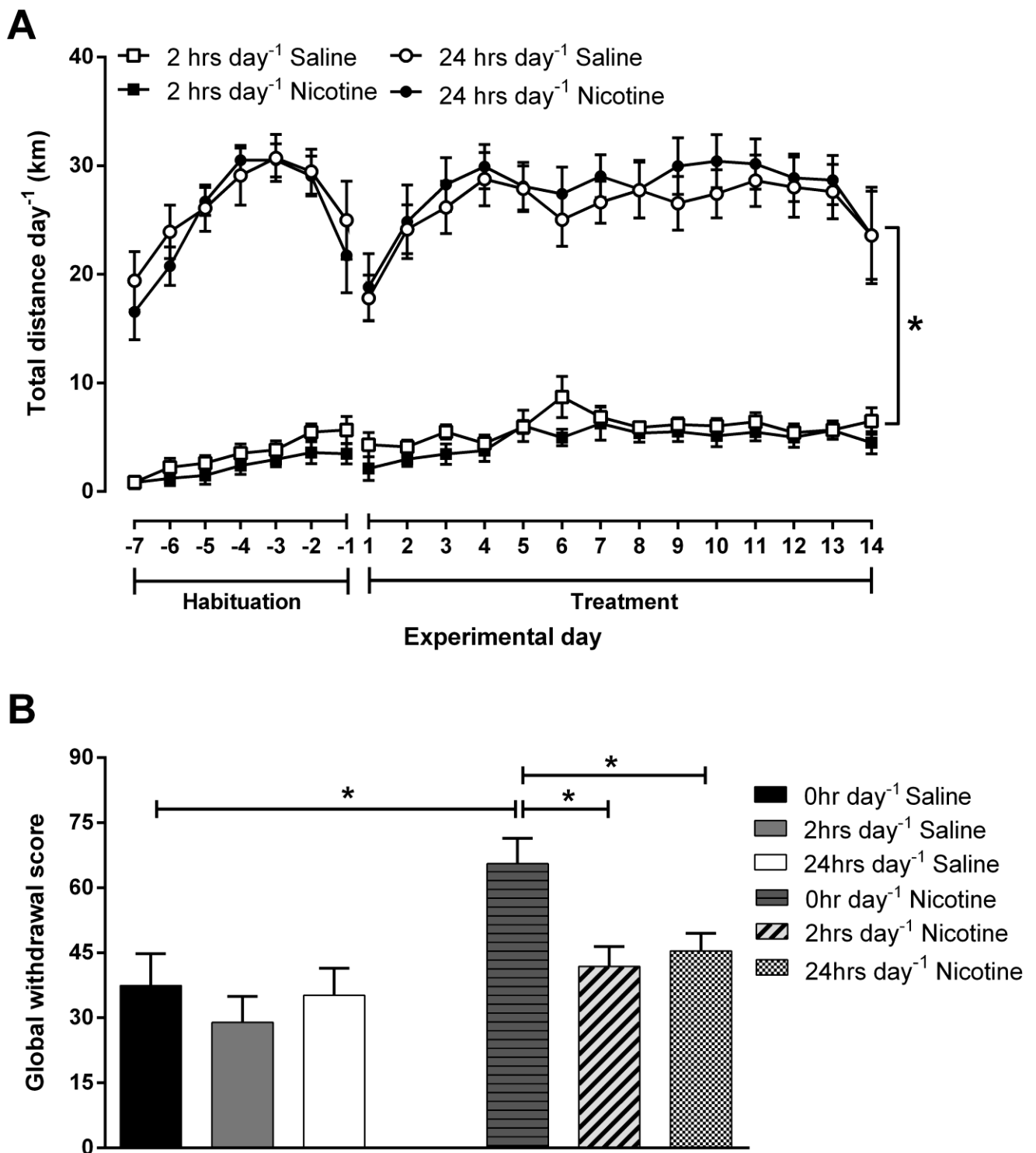
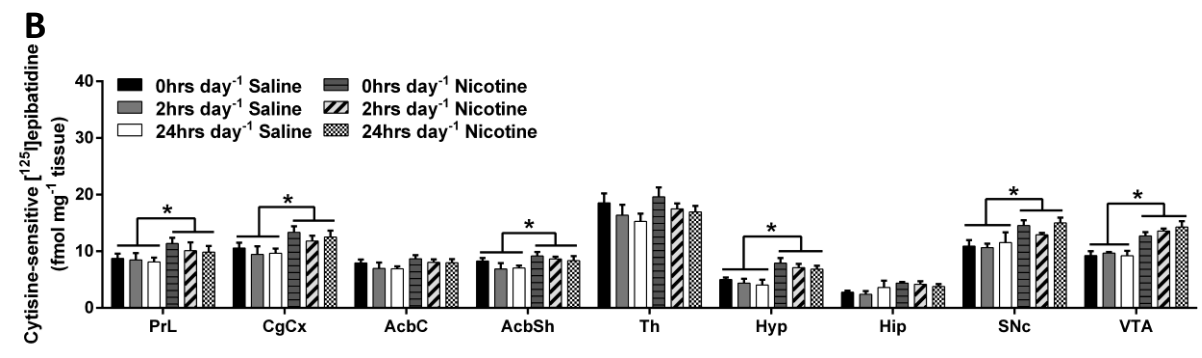
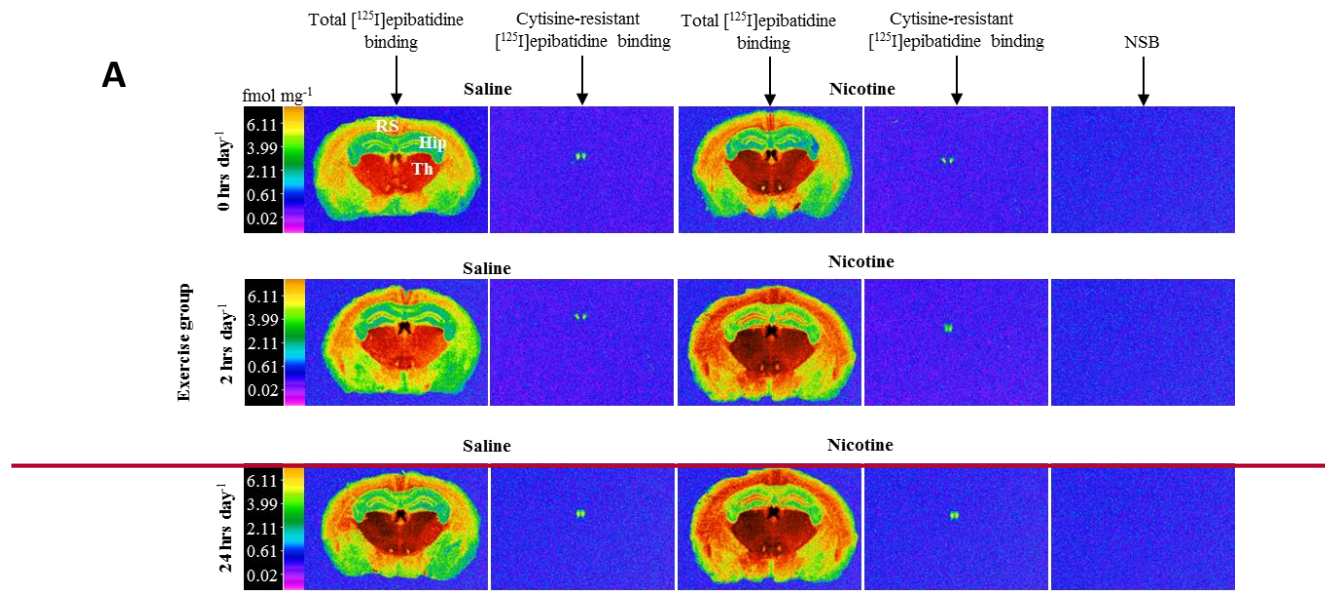


Figure 1. Effect of wheel-running exercise regimen on severity of nicotine withdrawal syndrome. Mice underwent one of three exercise regimens: 0, 2 or 24 hrs day⁻¹ running-wheel access. Withdrawal was precipitated by mecamylamine (3 mg kg⁻¹, s.c.) following 14 days of either saline or nicotine (24 mg kg⁻¹ day⁻¹) treatment via subcutaneous minipumps. (A) Total wheel-running activity during habituation and treatment phases of the experiment. Wheel-running activity was recorded and

converted into distance run day⁻¹ during the 7-day habituation and 14-day treatment periods. **(B)** Data for individual withdrawal symptoms were combined to give a total withdrawal measure. Data are presented as mean \pm SEM. * p <0.05. Precise group sizes are reported in Table 1.



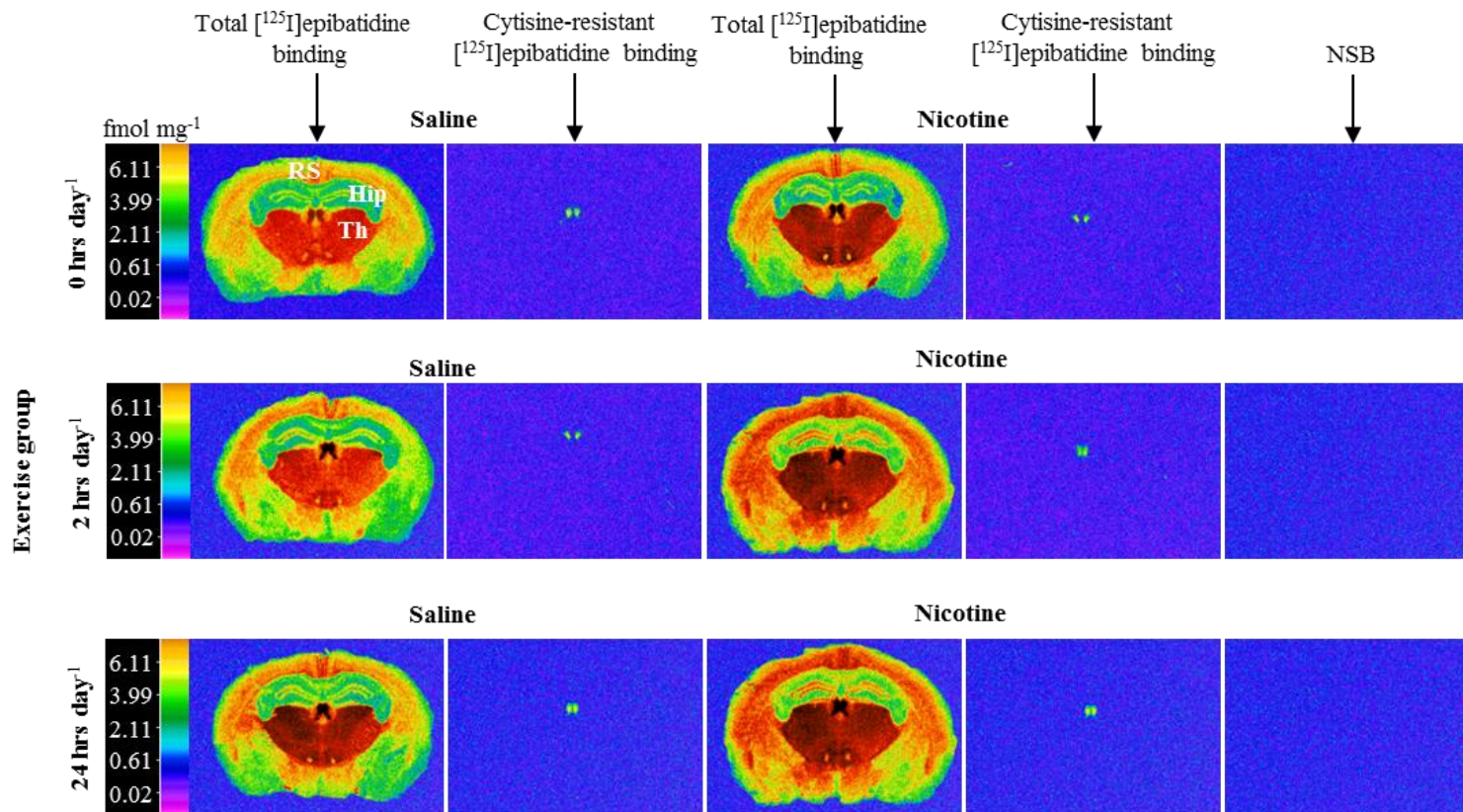
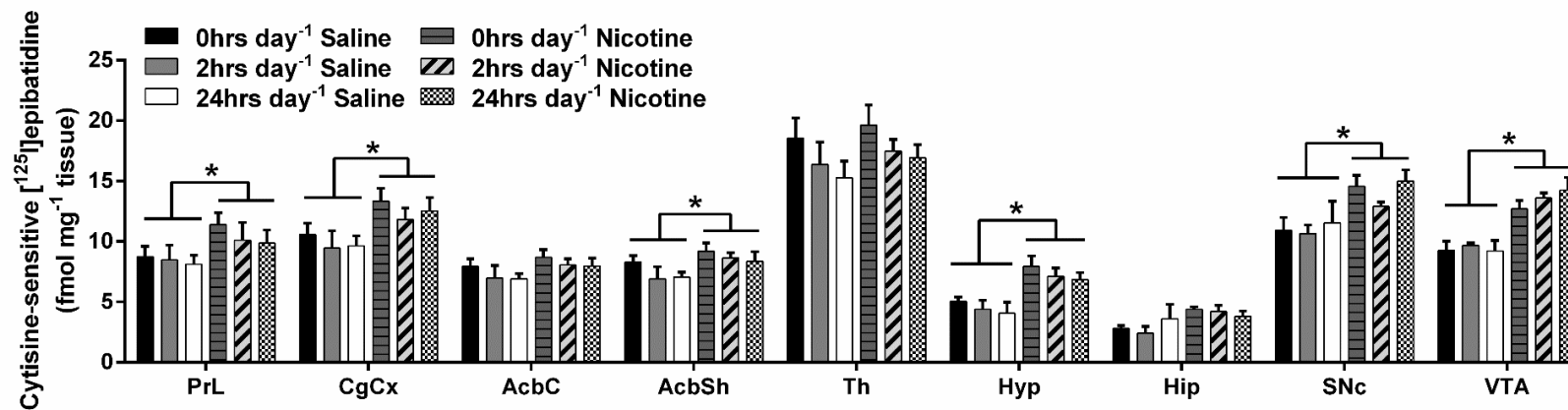
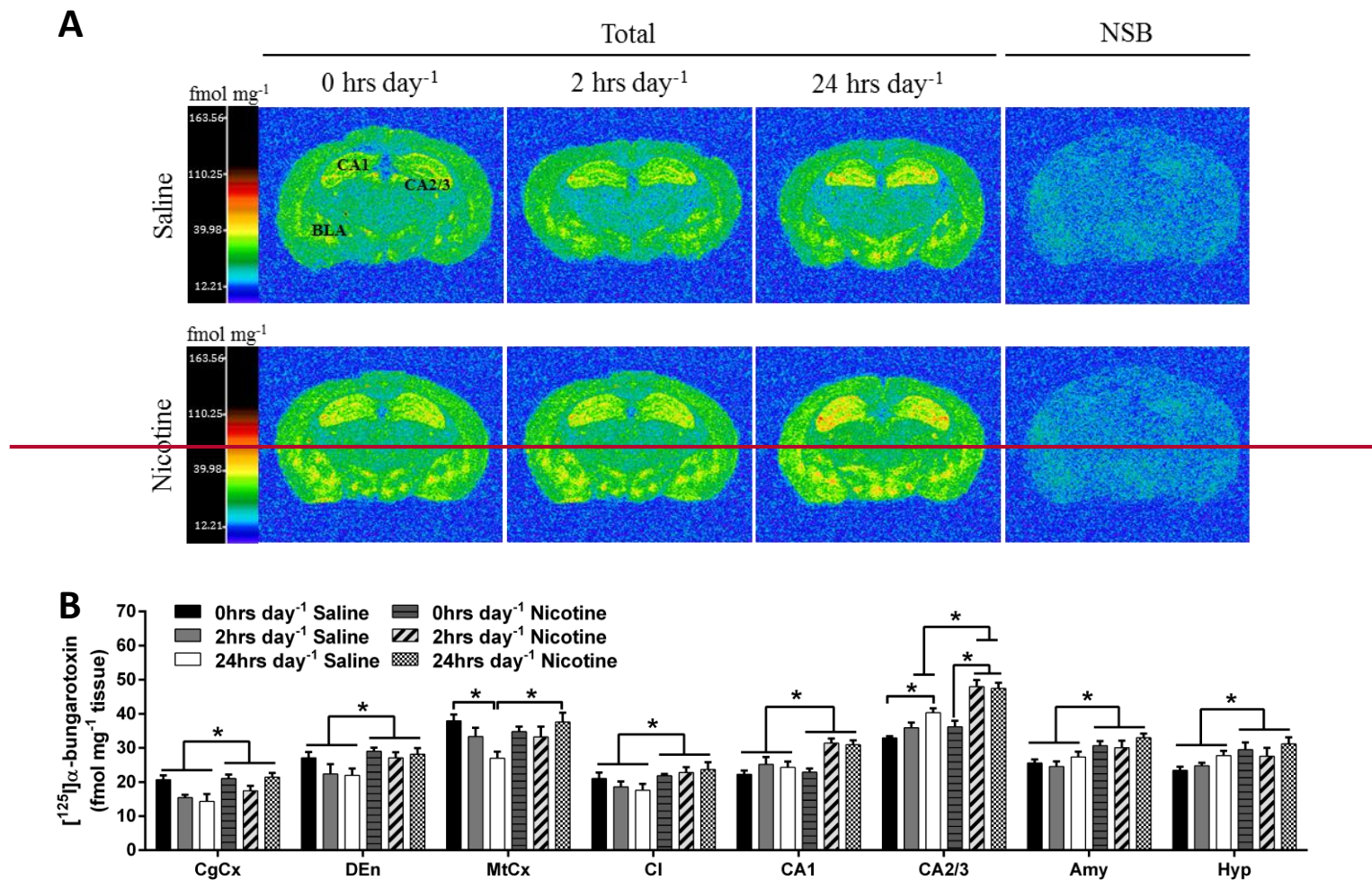
A**B**

Figure 2. Effect of exercise on $\alpha 4\beta 2^*$ nAChR binding in saline- and nicotine-withdrawn mice (A) Computer-enhanced colour autoradiograms of total and cytisine-resistant [125 I]epibatidine binding in coronal brain sections of C57Bl/6 mice treated with saline or nicotine (24 mg kg $^{-1}$ day $^{-1}$) via subcutaneous minipumps for 14 days, followed by mecamylamine-precipitated (3 mg kg $^{-1}$) withdrawal. Mice underwent one of three exercise regimes: 0, 2 or 24 hrs day $^{-1}$ running wheel access in their home cage. Coronal brain sections are shown cut at the level of the dorsal hippocampus and thalamus (Bregma -1.46 mm). The calibration bar presents pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. (B) Cytisine-sensitive [125 I]epibatidine binding in saline- and nicotine-withdrawn mice undergoing different exercise regimes in cortical brain regions. Data are presented as mean \pm SEM. * p <0.05. Precise group sizes are reported in Table 1. *Abbreviations:* AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; CgCx, cingulate cortex; Hip, hippocampus; Hyp, hypothalamus; SNc, substantia nigra pars compacta, Th, thalamus; VTA, ventral tegmental area.



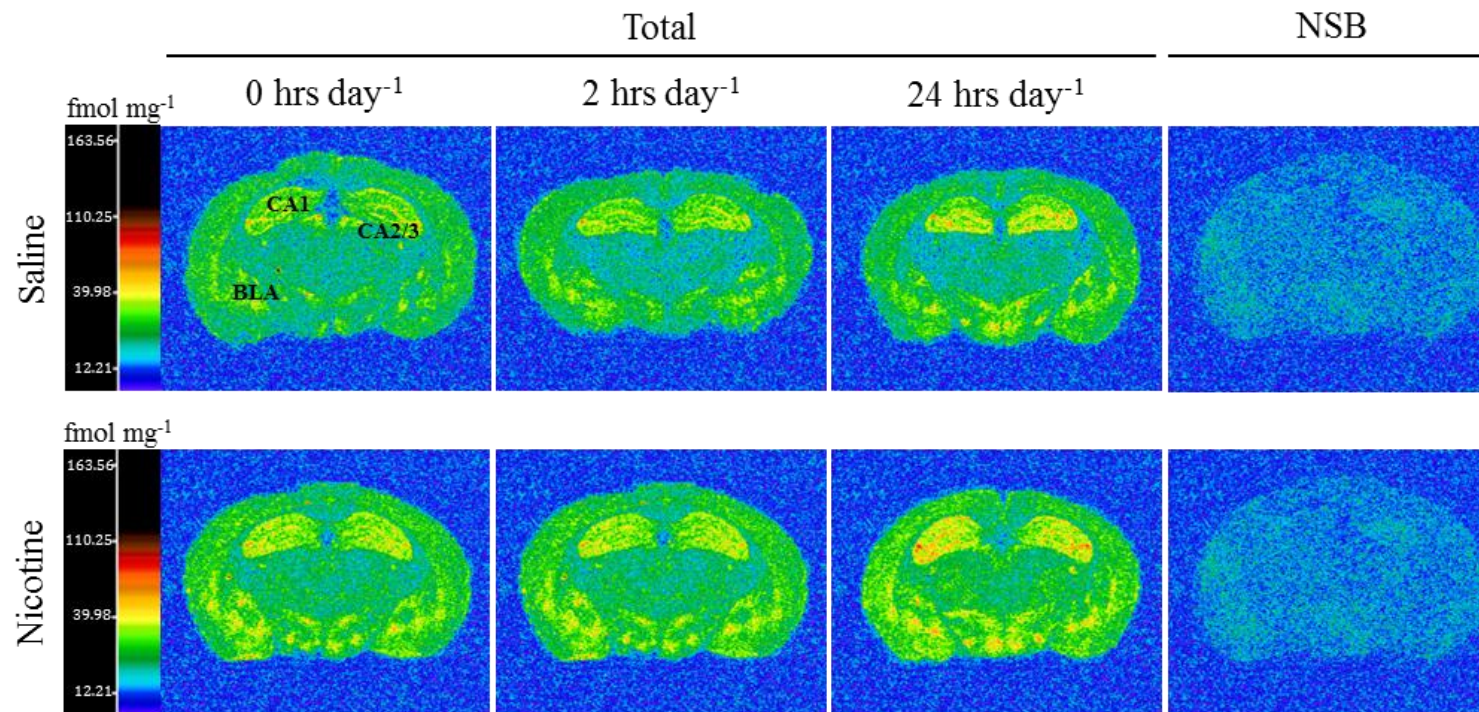
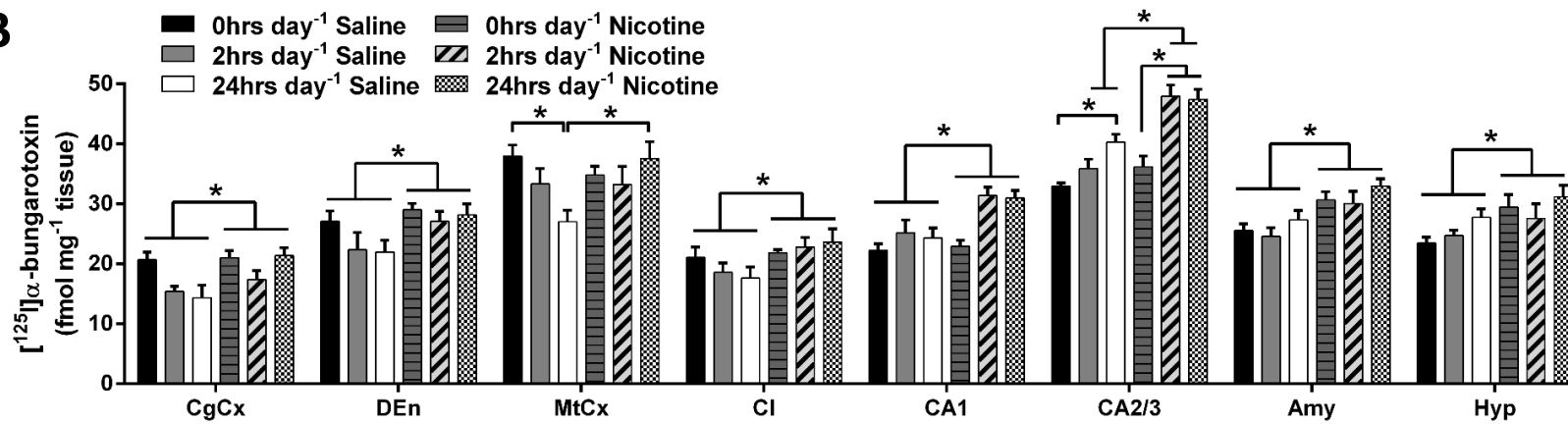
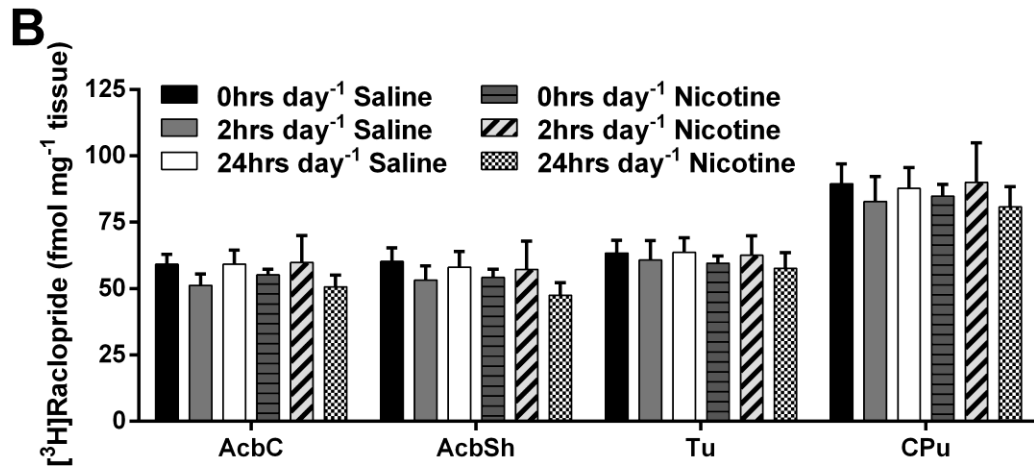
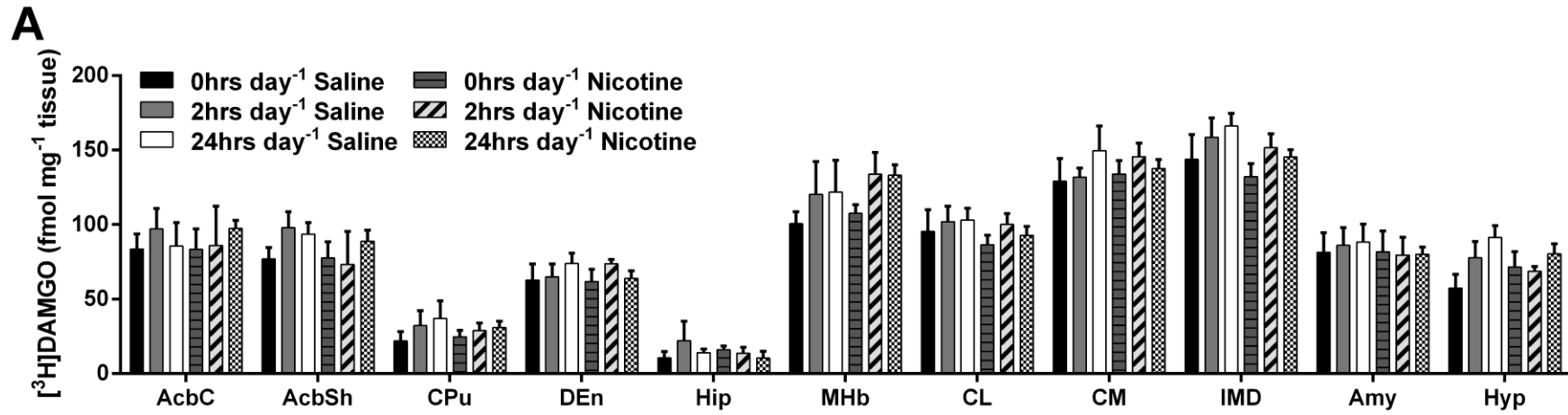
A**B**

Figure 3. Effect of exercise on $\alpha 7$ nAChR binding in saline- and nicotine-withdrawn mice. (A) Computer-enhanced colour autoradiograms of total [125 I] α -bungarotoxin and non-specific (NSB) binding in coronal brain sections of C57Bl/6 mice treated with chronic saline or nicotine via subcutaneous minipumps, followed by mecamylamine-precipitated (3 mg kg $^{-1}$) withdrawal. Mice underwent one of three exercise regimes: 0, 2 or 24 hrs day $^{-1}$ running wheel access in their home cage. Coronal brain sections are shown cut at the level of the dorsal hippocampus and thalamus (Bregma -1.46 mm). The calibration bar presents pseudo-colour interpretation of black and white film images in fmol/mg tissue equivalent. (B) Quantitative [125 I] α -bungarotoxin binding in saline- and nicotine-withdrawn mice undergoing different exercise regimes. Data are presented as mean \pm SEM. * p <0.05. Precise group sizes are reported in Table 1. *Abbreviations:* Amy, amygdala; CgCx, cingulate cortex; CA1, CA1 layer of the hippocampus; CA2/3, CA2 and CA3 layers of the hippocampus; Cl, claustrum; DEn, dorsal endopiriform; Hyp, hypothalamus; MtCx, motor cortex.



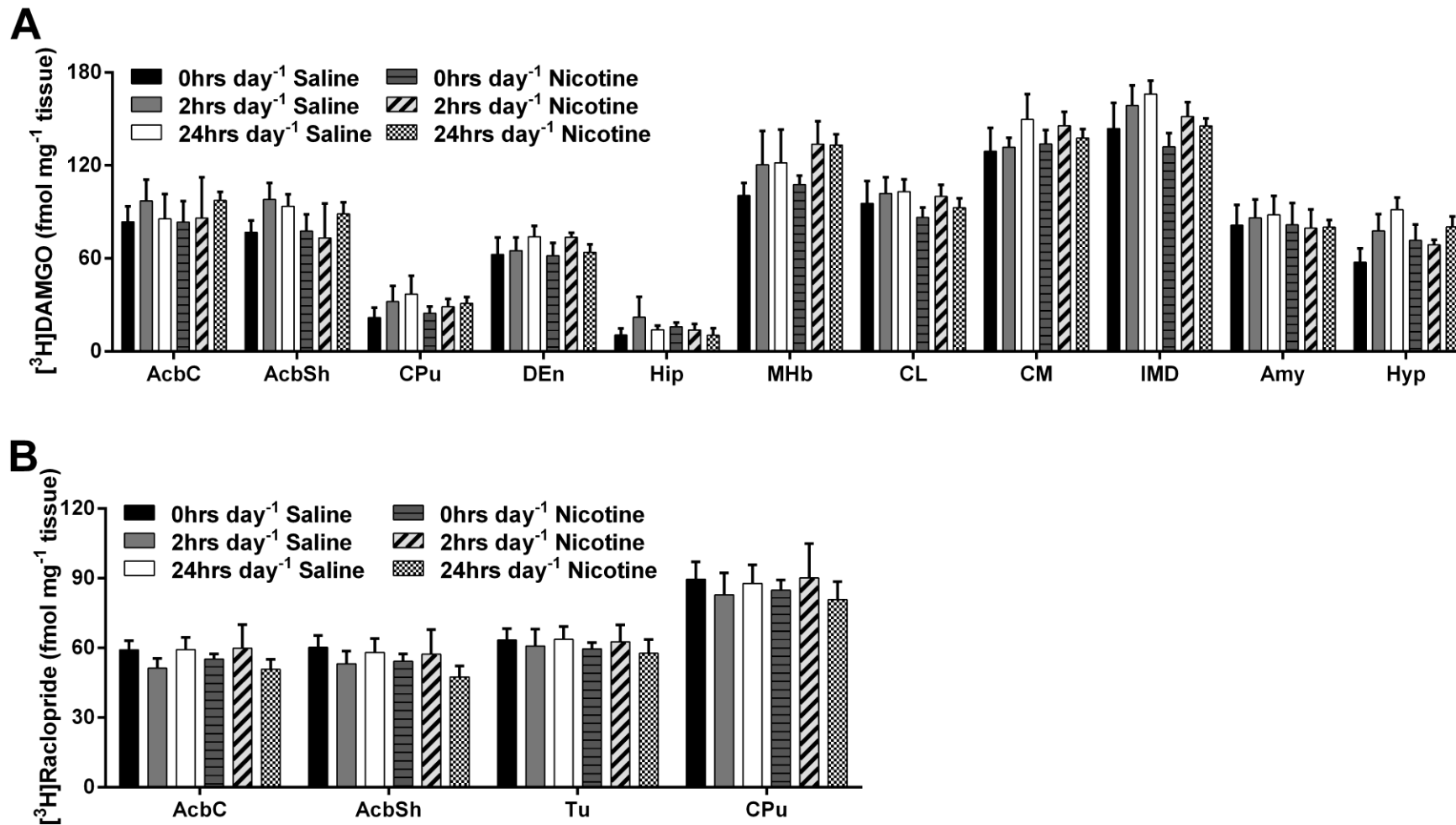


Figure 4. Effect of exercise on μ - and D2 receptor binding in saline- and nicotine-withdrawn mice. (A) $[^3\text{H}]\text{DAMGO}$ binding in non-cortical regions of saline- and nicotine-withdrawn mice undergoing different exercise regimes: 0, 2 or 24 hrs day⁻¹ running wheel access in their home cage. (B) $[^3\text{H}]\text{Raclopride}$ binding of saline- and nicotine-withdrawn mice undergoing different exercise regimes: 0, 2 or 24 hrs day⁻¹ running wheel access in their home cage. Data are presented as mean \pm SEM. Precise group sizes are reported in Table 1. *Abbreviations:* AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Amy, amygdala; CL, centrolateral thalamic nuclei; CM, centromedial thalamic nuclei; CPu, caudate putamen; DEn, dorsal endopiriform; Hip, hippocampus; Hyp, hypothalamus; IMD, intermediate thalamic nuclei; MHb, medial habenula; Tu, tubercle.

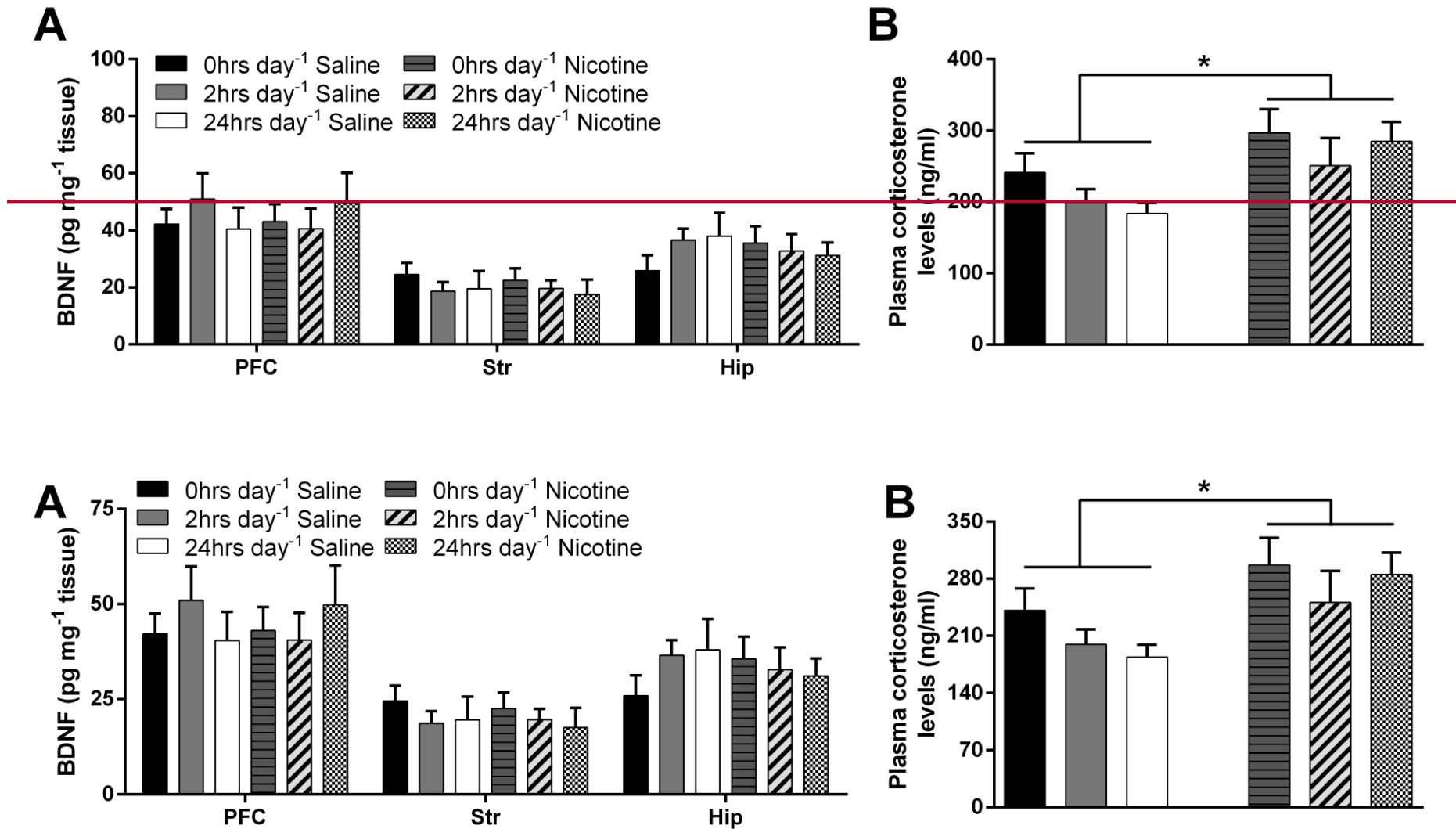


Figure 5. Effect of exercise on brain BDNF and plasma corticosterone levels in saline- and nicotine-withdrawn mice. (A) Brain-derived neurotrophic factor (BDNF) levels in saline- and nicotine-withdrawn mice undergoing different exercise regimes. Total BDNF levels from acid-withdrawn samples were determined using an enzyme-linked radioimmunoassay for nicotine- and saline-withdrawn mice undergoing 0, 2 or 24 hrs day⁻¹ running wheel access. (B) Plasma corticosterone levels in saline- and nicotine-withdrawn mice undergoing different exercise regimes. Plasma corticosterone content was determined using a [¹²⁵I] radioimmunoassay for

nicotine- and saline-withdrawn mice undergoing 0, 2 or 24 hrs day⁻¹. Data are presented as mean \pm SEM. * p <0.05. Precise group sizes are reported in Table 1.
Abbreviations: Hip, hippocampus; PFC, prefrontal cortex; Str, striatum.

Tables

Table 1: Statistical Analyses

	Sample size (figure order)	Factorial effects		Interaction effects	
Overall effects for Figure 1					
Running wheel activity					
Habituation	n=14,12,13,13	Factor 'treatment' F _[1,46] =0.784; p>0.05 Factor 'exercise' F _[1,46] =304.1; p<0.05*		Factor 'time (days)' F _[6,276] =1.350 p>0.05 Factor 'exercise' x 'time' F _[1,46] =0.001 p>0.05	
Treatment	n=14,12,13,13	Factor 'treatment' F _[1,47] =0.010; p>0.05 Factor 'exercise' F _[1,47] =204.1; p<0.05*		Factor 'time (days)' F _[13,611] =7.392 p<0.05* Factor 'exercise' x 'time' F _[13,611] =0.708 p>0.05	
Withdrawal score	n=13,14,13,12,12,13	H _[2] =8.940 [†] p<0.05*		Factor 'treatment' x 'time' F _[6,276] =0.590 p>0.05 Factor 'treatment' x 'exercise' x 'time' F _[6,276] =0.515 p>0.05 Factor 'treatment' x 'time' F _[13,611] =0.246 p>0.05 Factor 'treatment' x 'exercise' x 'time' F _[13,611] =0.365 p>0.05	
Overall effects for Figure 2					
a4β2* nAChR binding					
PrL	n=5,5,6,5,5,6	Factor 'treatment' F _[1,25] =5.362 p<0.05*		Factor 'exercise regimen' F _[2,25] =0.575 p>0.05	
CgCx	n=5,5,6,5,5,6	F _[1,25] =9.144 p<0.05*		F _[2,25] =0.738 p>0.05	
AcbC	n=5,5,6,5,5,6	F _[1,25] =2.415 p>0.05		F _[2,25] =1.017 p>0.05	
AcbSh	n=5,5,6,5,5,6	F _[1,25] =5.199 p<0.05*		F _[2,25] =1.391 p>0.05	
Th	n=5,5,6,5,5,6	F _[1,25] =1.101 p>0.05		F _[2,25] =2.243 p>0.05	
Hyp	n=5,5,6,5,5,6	F _[1,25] =21.10 p<0.05*		F _[2,25] =1.051 p>0.05	
Hip	n=5,5,6,5,5,6	F _[1,25] =4.198 p>0.05		F _[2,25] =0.158 p>0.05	
SNC	n=5,5,6,5,5,6	F _[1,25] =11.51 p<0.05*		F _[2,25] =0.881 p>0.05	
VTA	n=5,5,6,5,5,6	F _[1,25] =39.09 p<0.05*		F _[2,25] =0.546 p>0.05	
Overall effects for Figure 3					
a7 nAChR binding					
CgCx	n=5,5,6,5,5,6	Factor 'treatment' F _[1,25] =6.588 p<0.05*		Factor 'exercise regimen' F _[2,25] =4.357 p<0.05*	
DEn	n=5,5,6,5,5,6	F _[1,25] =7.059 p<0.05*		F _[2,25] =1.719 p>0.05	
MtCx	n=5,5,6,5,5,6	F _[1,25] =1.618 p>0.05		F _[2,25] =1.740 p>0.05	
Cl	n=5,5,6,5,5,6	F _[1,25] =6.661 p<0.05*		F _[2,25] =0.141 p>0.05	
CA1	n=5,5,6,5,5,6	F _[1,25] =13.30 p<0.05*		F _[2,25] =8.299 p<0.05*	
CA2/3	n=5,5,6,5,5,6	F _[1,25] =36.63 p<0.05*		F _[2,25] =21.80 p<0.05*	
Amy	n=5,5,6,5,5,6	F _[1,25] =20.61 p<0.05*		F _[2,25] =2.138 p>0.05	
Hyp	n=5,5,6,5,5,6	F _[1,25] =8.764 p<0.05*		F _[2,25] =2.475 p>0.05	
Overall effects for Figure 4					
μ receptor binding					
AcbC	n=5,5,5,5,5,6	Factor 'treatment' F _[1,24] =0.0002 p>0.05		Factor 'exercise regimen' F _[2,24] =0.190 p>0.05	
AcbSh	n=5,5,5,5,5,6	F _[1,24] =1.094 p>0.05		F _[2,24] =0.807 p>0.05	
CPu	n=5,5,5,5,5,6	F _[1,24] =0.105 p>0.05		F _[2,24] =0.892 p>0.05	
DEn	n=5,5,5,5,5,6	F _[1,24] =0.013 p>0.05		F _[2,24] =0.539 p>0.05	
Hip	n=5,5,5,5,5,6	F _[1,24] =0.160 p>0.05		F _[2,24] =0.422 p>0.05	
MHb	n=5,5,5,5,5,6	F _[1,24] =0.647 p>0.05		F _[2,24] =1.329 p>0.05	
CL	n=5,5,5,5,5,6	F _[1,24] =0.953 p>0.05		F _[2,24] =0.626 p>0.05	
CM	n=5,5,5,5,5,6	F _[1,24] =0.057 p>0.05		F _[2,24] =0.602 p>0.05	
IMD	n=5,5,5,5,5,6	F _[1,24] =2.465 p>0.05		F _[2,24] =1.891 p>0.05	
Amy	n=5,5,5,5,5,6	F _[1,24] =0.251 p>0.05		F _[2,24] =0.027 p>0.05	
Hyp	n=5,5,5,5,5,6	F _[1,24] =0.070 p>0.05		F _[2,24] =3.205 p>0.05	
D₂ receptor binding					
AcbC	n=5,5,6,5,5,6	Factor 'treatment' F _[1,25] =0.097 p>0.05		Factor 'exercise regimen' F _[2,25] =0.102 p>0.05	
AcbSh	n=5,5,5,5,5,6	F _[1,25] =0.744 p>0.05		F _[2,25] =0.320 p>0.05	
Tu	n=5,5,5,5,5,6	F _[1,25] =0.328 p>0.05		F _[2,25] =0.017 p>0.05	
CPu	n=5,5,5,5,5,6	F _[1,25] =0.043 p>0.05		F _[2,25] =0.066 p>0.05	
Overall effects for Figure 5					
BDNF levels					
PFC	n=8,7,9,8,7,7	Factor 'treatment' F _[1,40] =0.0003 p>0.05		Factor 'exercise regimen' F _[2,40] =0.089 p>0.05	
Str	n=8,7,9,8,7,7	F _[1,40] =0.089 p>0.05		F _[2,40] =0.796 p>0.05	
Hip	n=8,7,9,8,7,7	F _[1,40] =0.003 p>0.05		F _[2,40] =0.335 p>0.05	
Corticosterone levels					
Plasma	n=7,8,7,7,7,8	F _[1,34] =9.757 p<0.05*		F _[2,34] =1.429 p>0.05	
Overall effects for Supplementary Figure S1					
a4β2* nAChR binding					
MHb (cytisine-sensitive)	n=5,5,5,5,5,6	Factor 'treatment' F _[1,26] =0.388 p>0.05		Factor 'exercise regimen' F _[2,26] =1.848 p>0.05	
MHb (cytisine-resistant)	n=5,5,5,5,5,6	F _[1,52] =0.060 p>0.05		F _[2,52] =0.0690 p>0.05	
Overall effects for Supplementary Table S1					
a4β2* nAChR binding					

FrA	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=25.34$	$p<0.05^*$	$F_{[2,25]}=0.063$	$p>0.05$	$F_{[2,25]}=1.479$	$p>0.05$
MtCx	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=13.23$	$p<0.05^*$	$F_{[2,25]}=1.304$	$p>0.05$	$F_{[2,25]}=0.287$	$p>0.05$
SS	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=24.82$	$p<0.05^*$	$F_{[2,25]}=0.589$	$p>0.05$	$F_{[2,25]}=0.150$	$p>0.05$
Pir	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=29.42$	$p<0.05^*$	$F_{[2,25]}=0.504$	$p>0.05$	$F_{[2,25]}=0.185$	$p>0.05$
RS	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=6.512$	$p<0.05^*$	$F_{[2,25]}=0.589$	$p>0.05$	$F_{[2,25]}=0.778$	$p>0.05$
CPu	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=7.022$	$p<0.05^*$	$F_{[2,25]}=0.624$	$p>0.05$	$F_{[2,25]}=0.010$	$p>0.05$
MS	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=16.50$	$p<0.05^*$	$F_{[2,25]}=0.709$	$p>0.05$	$F_{[2,25]}=0.651$	$p>0.05$
VDB	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=15.93$	$p<0.05^*$	$F_{[2,25]}=0.266$	$p>0.05$	$F_{[2,25]}=0.650$	$p>0.05$
AuCx	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=24.89$	$p<0.05^*$	$F_{[2,25]}=0.470$	$p>0.05$	$F_{[2,25]}=0.236$	$p>0.05$

Overall effects for Supplementary Table S2

a7 nAChR binding

FrA	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=2.145$	$p>0.05$	$F_{[2,25]}=0.547$	$p>0.05$	$F_{[2,25]}=0.165$	$p>0.05$
CPu	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=1.564$	$p>0.05$	$F_{[2,25]}=1.327$	$p>0.05$	$F_{[2,25]}=0.501$	$p>0.05$
ZI	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=2.275$	$p>0.05$	$F_{[2,25]}=1.264$	$p>0.05$	$F_{[2,25]}=0.718$	$p>0.05$
VLG	<i>n</i> =5,5,6,5,5,6	$F_{[1,25]}=3.710$	$p>0.05$	$F_{[2,25]}=1.302$	$p>0.05$	$F_{[2,25]}=2.215$	$p>0.05$

Data were analysed with ANOVA unless otherwise indicated, with significance threshold of $*p<0.05$.

† Non-parametric Kruskal-Wallis test, with significance threshold of $*p<0.05$.

Abbreviations: AcbC, nucleus accumbens core; AcbSh, nucleus accumbens shell; Amy, amygdala; AuCx, auditory cortex; CA1, CA1 area of the hippocampus; CA2/3, CA2 and CA3 areas of the hippocampus; CgCx, cingulate cortex; Cl, clostrum; CL, centrolateral thalamic nuclei; CM, centromedial thalamic nuclei; CPu, caudate-putamen; D₂ receptor, dopamine D₂ receptor; DEn, dorsal endopiriform; FrA, frontal association; Hip, hippocampus; Hyp, hypothalamus; IMD, intermediate thalamic nuclei; MHb, medial habenula; MS, medial septum; MtCx, motor cortex; nAChR, nicotinic acetylcholine receptor; Pir, piriform cortex; PrL, prelimbic cortex; RS, retrosplenial cortex; SNc, Substantia nigra pars compacta; SS, somatosensory cortex; Th, thalamus; Tu, olfactory tubercle; VDB, vertical limb of the diagonal band of Broca; VLG, ventral lateral geniculate; VTA, ventral tegmental area; ZI, zona incerta.