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ARTICLE

Altered neuronal activity in the ventromedial prefrontal cortex drives nicotine intake escalation

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Nicotine addiction develops after prolonged drug use and escalation of drug intake. However, because of difficulties in demonstrating escalation of nicotine use in rats, its underlying neuroadaptations still remain poorly understood. Here we report that access to unusually high doses of nicotine (i.e., from 30 µg to 240 µg/kg/injection) for self-administration precipitated a rapid and robust escalation of nicotine intake and increased the motivation for the drug in rats. This nicotine intake escalation also induced long-lasting changes in vmPFC neuronal activity both before and during nicotine self-administration. Specifically, after escalation of nicotine intake, basal vmPFC neuronal activity increased above pre-escalation and control activity levels, while ongoing nicotine self-administration restored these neuronal changes. Finally, simulation of the restoring effects of nicotine with in vivo optogenetic inhibition of vmPFC neurons caused a selective de-escalation of nicotine self-administration.

Neuropsychopharmacology (2023) 48:887–896; https://doi.org/10.1038/s41386-022-01428-9

INTRODUCTION

Nicotine is the main substance responsible for tobacco addiction which remains the most prevalent addiction in the world today and a leading cause of preventable morbidity and mortality worldwide [1]. Among people who smoke tobacco cigarettes, many escalate their daily consumption [2] and transition to a state of tobacco addiction [3–6]. In rodents, escalation of drug use is typically observed with prolonged daily access to several intravenous drugs—including cocaine [7–9], heroin [9–14] and methamphetamine [15–18]. In contrast, unless subjected to different access conditions [19, 20], rats do not show nicotine intake escalation following prolonged access to the drug [21, 22]. The neuroadaptations underlying escalation of nicotine intake therefore still remain poorly understood.

Clinical neuroscience research has shown that the ventromedial prefrontal cortex (vmPFC) is important for controlling and inhibiting drug seeking and motivation [23]. Specifically, neuroimaging studies have consistently found changes in the vmPFC in chronic cigarette smokers [24–28], notably a decrease in gray matter volume and in basal neuronal activity. These changes could explain why they experience difficulty in controlling their consumption of cigarettes when they want to quit or cut down. Moreover, preclinical research on animal models of drug addiction also supports an involvement of the vmPFC in drug-taking and -seeking behaviors [29–34]. For instance, vmPFC inhibition reduces methamphetamine and heroin seeking after extinction or cocaine seeking after abstinence [29, 32, 35, 36]. However, we do not know how the activity of vmPFC neurons is modified during prolonged access to nicotine self-administration and, if so, whether these alterations contribute to the development and maintenance of nicotine intake escalation.

Here we set out to address these questions by recording in vivo vmPFC neuronal activity in a newly developed rat model of nicotine intake escalation. First, we report that access to unusually high doses of nicotine (i.e., from 30 µg to 240 µg/kg/injection) can precipitate a rapid and robust escalation of intake that was associated with an increased motivation to take the drug. Such escalation of nicotine intake induces long-lasting changes in the basal activity of vmPFC neurons measured 24 h after the last self-administration session and also in their activity during ongoing intravenous nicotine self-administration. Specifically, after escalation of nicotine intake, basal vmPFC neuronal activity increased above pre-escalation and control activity levels, while in contrast nicotine intake during ongoing nicotine self-administration restored these neuronal changes. Finally, simulation of the restoring effects of nicotine using in vivo optogenetic inhibition of vmPFC neurons caused a selective de-escalation of nicotine self-administration. Overall, these data suggest that part of the motivation for using nicotine during escalation might be to restore post-escalation changes in vmPFC basal neuronal activity.

MATERIAL AND METHODS

Subjects

A total of 88 adult male Wistar rats were used (225–250 g at the beginning of experiments, Charles River, Lyon, France). Two rats did not complete the experiment because of loss of catheter patency, thereby leaving 78 rats for final analysis (39 for the nicotine escalation experiment, 10 for the in vivo electrophysiology experiment, and 37 for the optogenetic experiment). Rats were housed in groups of 2 and were maintained in a light- (reverse light-dark cycle), humidity- (60 ± 20%) and temperature-controlled vivarium (21 ± 2 °C), with water and food available ad libitum. All behavioral testing occurred during the dark phase of the light-dark cycle. Home cages were enriched with a nylon gnawing bone and a cardboard tunnel (Plexx BV, The Netherlands). All experiments were carried out in accordance with institutional and international standards of care and use of laboratory

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Received: 24 March 2022 Revised: 4 August 2022 Accepted: 6 August 2022
Published online: 30 August 2022

animals [UK Animals (Scientific Procedures) Act, 1986; and associated guidelines; the European Communities Council Directive (2010/63/UE, 22 September 2010) and the French Directives concerning the use of laboratory animals (décret 2013–118, 1 February 2013)]. The animal facility has been approved by the Committee of the Veterinary Services Gironde, agreement number B33–063–922.

Nicotine self-administration and escalation procedures

Fourteen identical operant chambers (30 × 40 × 36 cm) were used for nicotine self-administration testing and training (Imétronic, Pessac, France). Each chamber was equipped with two retractable metal levers on opposite panels of the chamber and a corresponding white cue light above each lever. Under deep anesthesia (mixture of ketamine 100 mg/kg and xylazine 15 mg/kg, i.p), rats were surgically prepared with an indwelling silastic catheter (Dow Corning Corporation, Michigan, USA) in the right jugular vein that exited the skin in the middle of the back about 2 cm below the scapulae. After surgery, rats were flushed daily with 0.2 ml of an ampicillin solution (0.1 g/ml) containing heparin (300 IU/ml) to maintain patency. After 7 days of recovery, rats were first habituated during 2–3 h daily sessions to the experimental chambers with no lever or light cue presented. Then rats were trained to press a lever to self-administer a unit dose of nicotine (30 µg/kg/injection free base; 40 µl in 1 s) under a final fixed-ratio (FR) 1 schedule of reinforcement during 3 h daily sessions. Self-administration sessions were run 5–6 days/week. All self-administration sessions began with extension of the operant lever and ended with its retraction after 3 h. Intravenous delivery of nicotine began immediately after completion of the lever press and was accompanied by illumination of the light cue above the lever for 20 s. Responses during the light cue were recorded but had no programmed consequence.

Rats trained to self-administer nicotine for 15 days were then divided in two groups: one no-escalation (No ES, $n = 12$) and one escalation groups (ES, $n = 27$). In the no-escalation group, the unit dose of nicotine remained at the initial dose of 30 µg/kg/injection for 30 additional sessions, while in the escalation group the unit dose of nicotine available progressively increased to 120 µg and then to 240 µg/kg/injection for 15 sessions at each dose. Unit doses of nicotine were presented in ascending order to reduce the risk of accidental overdose or overconsumption with subsequent aversive reactions to the drug [37]. This increase in the unit dose of nicotine available was done by increasing the injection volume (to 160 and 320 µl), a procedure known to speed up the escalation process [10, 38]. Twenty-four hours after the last escalation day, rats were tested under a progressive ratio schedule of reinforcement during which the response requirement for nicotine reinforcement was increased according to the following sequence: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, etc [39]. The progressive ratio session ended after 1 h had elapsed without a drug delivery reward or after a maximum of 6 h. The last ratio completed during the session, called the breaking point, was used to measure the intensity of drug seeking. Rats were then exposed to 3 additional self-administration session days (either 30 µg or 240 µg/kg/injection according to the group) and then to a single sensitization test session during which all rats were exposed to the unit dose of 30 µg/kg/injection.

Nicotine-induced behavioral excitement was observed and evaluated after the first nicotine injection using a four point-ranked score (0: no effect; 1: mild head tremor and straub tail; 2: apparent tremors in extended regions, 3: severe tremors and convulsions-like responses) [40] during the 15 days of nicotine self-administration at the highest dose of 240 µg/kg/injection. Both the incidence and the behavioral score were quantified across days and compared using a Chi X^2 or an ANOVA respectively.

Sucrose self-administration

Eight identical operant chambers (30 × 40 × 36 cm) were used for sucrose self- administration (Imétronic, Pessac, France). Each chamber was equipped with one retractable metal lever, a white cue light above the lever and a drinking cup nearby. Rats were trained to lever press for sucrose (i.e., 0.28 ml of 10% sucrose over 10 s) under a FR 1 time-out 10 s schedule of reinforcement during 14 daily sessions before optogenetic testing. Each session began with extension of the operant lever and ended after a maximum of 40 rewards or after 1 h, whatever came first. Responses during the light cue were recorded but had no programmed consequence.

In vivo electrophysiological recordings

To assess whether nicotine intake escalation altered vmPFC neuronal activity, we used in vivo electrophysiology to record single-unit activity in the vmPFC. An independent group of rats (no-escalation and escalation, $n = 5$ for each group) were surgically prepared with a catheter in the right jugular vein and an array of 16 teflon-coated stainless steel microwires (MicroProbes Inc, Gaithersburg, MD) in the vmPFC [AP:+2.5–+4.5 mm, ML: 0.3–1.2 mm, and DV: −4.8 mm relative to skull level], as previously described [41].

Voltage signals from each microwire array were band-pass-filtered, such that activity between 150 and 8000 Hz was analyzed as spiking activity. Data were amplified, processed and digitally captured using commercial hardware and software (Plexon Inc, Dallas, TX). Single units were discriminated with principal component analysis (Offline sorter, Plexon Inc, Dallas, TX). The quality of recorded units was ensured with an interspike interval criterion (>1 ms) and a signal:noise criterion (>3X noise band). Neurons were classified into putative interneurons and pyramidal neurons according to the waveform spike width and average firing rate. Waveforms with durations shorter than 300 µs were classified as putative interneurons, and waveforms with durations longer than 300 µs were classified as putative pyramidal neurons, as previously described [41, 42]. We recorded a total number of putative interneurons ($n = 14$) and pyramidal neurons ($n = 240$) across all recordings days. Due to the small number of interneurons, data analysis exclusively focused on putative pyramidal neurons. We analyzed a total 81 pyramidal neurons during Habituation, 79 neurons during Initial self-administration and 80 neurons during Final self-administration. Electrophysiological data were analyzed using NeuroExplorer (Plexon Inc., Dallas, TX).

After 1 week of recovery, rats were trained through the same nicotine intake escalation as described above. Electrophysiological recordings were conducted several times for each individual rat on the following days: on the habituation day before escalation (Hab), on the last day of nicotine self-administration acquisition (30 µg/kg/injection for both no-escalation and escalation groups; Initial SA), and on the last day of nicotine self-administration escalation (30 µg/kg/injection and 240 µg/kg/injection for both no-escalation and escalation respectively; Final SA). Recording sessions were identical to self-administration sessions except that they also included an initial 30 min pre-drug period—during which the chamber was dark and no session events occurred—to allow measurement of basal tonic neuronal activity before onset of nicotine intake [42]. Tonic vmPFC neurons firing rates were measured during the 30 min pre-drug period before onset of nicotine self-administration (i.e., Before SA) and during the 3 h nicotine self-administration session (i.e., During SA) and normalized using the transformation of $\log_{10}(x + 1)$ to reduce the skew of the firing rate distribution. Tonic neuronal activity was determined for each individual rat and averaged across rats. Individual neurons were tested for a significant and stable session-change in firing (increase or decrease) during the 3 h nicotine self-administration session relative to the pre-drug period using a Mann-Whitney test [42, 43]. Neurons were also tested for phasic long-duration change in firing (increase or decrease) that occurred slowly after, and relative to, firing in the 2 min before the nicotine reinforced lever press [44]. At the end of the study, histological procedures were used to identify the location of all wire tips used to record neurons. Coronal sections showing the location of microwires electrodes tips from which vmPFC neurons were recorded are shown in Fig. 3a.

In vivo optogenetic procedures

Viral infection of vmPFC pyramidal neurons was conducted in an independent group of rats ($n = 26$) trained to self-administered nicotine. Then, under isoflurane anesthesia, light-gated opsins viruses encoding the archaerhodopsin-3.0 protein (no-escalation and escalation, $n = 8$ for each group; CaMKII-α-eArchT3.0-eYFP; 5.5×10^{12} viral molecules/mL; UNC Vector Core) or a control virus (no-escalation and escalation, $n = 5$ for each group; CaMKII-eYFP 5.5×10^{12} viral molecules/mL; UNC Vector Core) were infected bilaterally in the vmPFC (AP:+3 mm; ML:±1.4 mm; DV: −4.5 from skull, angle 10°) to selectively infect pyramidal projecting neurons. A total of 0.5 µl/hemisphere was injected at a rate of 0.1 µl/min for 10 min using an Hamilton syringe mounted in an infusion pump after which the injector will be left in place for an additional 10 min to allow virus diffusion. Optic fibers (200/230 µm core diameter Plexon Inc., Dallas, TX) were implanted bilaterally slightly above the injection site (~0.3 mm) to ensure illumination of the transduced neurons and secured to the skull screws and dental cement. Seven days after surgery, rats were retrained to nicotine self-administration escalation for 20 additional days before being testing for optogenetic manipulations. Opto-inhibition of vmPFC neurons after nicotine escalation (250 ms pulse width at 1 Hz) [45] during the 10 min pre-drug period before the onset of nicotine self-administration was

assessed twice in two separate 1 h nicotine self-administration sessions (no light and light conditions, order counter-balanced between groups) at the lowest dose of 30 µg/kg/injection to ensure sufficient number of presses and injections. Each implanted optic fiber was connected to a LED module (550 nm) mounted on a dual LED commutator connected to an optogenetic controller (PlexBright, Plexon Inc., Dallas, TX). A separate group of rats ($n = 8$) was similarly injected with eArchT3.0 in the vmPFC and trained to self-administer sucrose 10% under FR1 for 14 days. Optogenetic inhibition of vmPFC neurons (250 ms pulse width at 1 Hz during the 10 min baseline period) was first assessed in two separate FR1 sucrose sessions (no light and light conditions, order counter-balanced). Then the FR was increased to 3 to further increase response rate in comparison to the lower rate during initial FR1 training, and opto-inhibition again assessed in two separate FR3 sucrose sessions (no light and light conditions, order counter-balanced). Efficiency of optical stimulation in inhibiting neuronal vmPFC pyramidal neurons activity was controlled in an independent group of rats injected with eArchT3.0 ($n = 3$) using optrodes by simultaneously recording in vivo vmPFC neuronal activity before and after optogenetic stimulation (250 ms pulse width at 1 Hz during 5 min). At the end of the experiments, rats were perfused transcardially with 4% paraformaldehyde. Brains were removed, serially cut on a cryostat (50 µm) and stored in PBS as floating sections for immunohistochemistry. Brains floating sections were first put in a blocking solution with Normal Donkey Serum 5% in PBS-Triton 0.3% (PBST) during 90 min and then incubated with rabbit anti-GFP antibody (1:2000, Invitrogen, A11122) and mouse anti-CAMKII antibody (1:500, Thermo Fisher, MA1–048) overnight at room temperature (RT). Sections were next washed with PBST and revealed with donkey anti-rabbit antibody conjugated to Alexa 488 (1:1000, Invitrogen, A21206) and donkey anti-mouse antibody conjugated to A568 (1:1000, Invitrogen, A10037) for 90 min at RT. Sections were finally mounted in antifading Vectashield medium with 4,6-diamidino-2-phenylindole DAPI (Vector Laboratories), coverslipped and imaged on an epifluorescence microscope (Olympus). Histological and immunological procedures were used to verify the opsin expression and identify the location of all optic fibers. Coronal sections showing the location of the tips of the optic fibers within the vmPFC are shown in Fig. 4a.

Data analysis

Data were subjected to one-way or two-way ANOVAs with repeated measures, followed by Tukey post hoc tests where relevant. For the optogenetic study, data were subjected to three-way ANOVAs with training condition (escalation or no-escalation) and virus type as between factors and light condition (light or no-light) as repeated measures, followed by Tukey post hoc tests where relevant. Percentage of neurons were compared using the two-proportion *z*-test. Statistical analyses were run using Statistica, version 7.1 (Statsoft Inc., Maisons-Alfort, France).

RESULTS

Robust escalation of nicotine intake in rats exposed to unusually high doses of nicotine self-administration

We first developed a new three stages model of nicotine intake escalation (see Material and Methods; Fig. 1). Briefly, one group of rats had access to nicotine self-administration at the unit dose of 30 µg/kg/injection (no-escalation group, No ES, $n = 12$), while in the other group the unit dose of nicotine available progressively increased from 30 µg to 120 µg and to 240 µg/kg/injection (escalation group, ES, $n = 27$). Though drug intake was similar in both groups at the initial dose of 30 µg/kg/injection, nicotine intake increased with the unit dose of nicotine ($F(44,1628) = 8.44$; $p < 0.001$; Fig. 1a). At the dose of 120 µg/kg/injection, all rats immediately raised their drug intake to reach within 3 days a stable level of nicotine intake that they maintained thereafter across daily sessions ($F(44,1144) = 19.7$; $p < 0.001$; 15th vs 16th session; $p < 0.01$ and 18th vs 30th session; NS; Fig. 1a). Next, when the dose was enhanced to the final highest dose of 240 µg/kg/injection, rats further increased their intake but did so gradually over time (31st vs 45th session; $p < 0.001$; Fig. 1a). In contrast, in the no-escalation group, nicotine intake increased at the beginning of the training but remained stable during the rest of the procedure ($F(44,484) = 4.41$; $p < 0.001$; 1st vs 7th session; $p < 0.05$ and 15th vs 45th session; *NS*). At the end of the escalation procedure, the level of nicotine intake (i.e., average over the last 3 sessions at each dose) was 3.5 times higher in the escalation group than in the no-escalation group ($F(2,74) = 19.7$; $p < 0.001$; Fig. 1b). Finally, rats in the escalation group showed a higher motivation to obtain nicotine as revealed both by elevated break point in a progressive-ratio reinforcement schedule ($F(2,74) = 19.7$; $p < 0.001$; Fig. 1c), and increased number of lever presses ($F(1,37) = 11.6$; $p < 0.001$; Fig. 1d) and increased number of nicotine injections when decreasing the nicotine dose back to 30 µg/kg/injection ($F(1,37) = 9.4$; $p < 0.01$; Fig. 1e). Moreover, this increase in nicotine intake was particularly evident during the first 10 min ($F(17,663) = 3.61$; $p < 0.001$; Fig. 1f), a drug-loading behavior that reflect an acquired need state for a higher level of nicotine intoxication.

During the course of escalation of nicotine intake, rats in the escalation group exposed to the highest dose of nicotine (240 µg/kg/injection) began to exhibit convulsive-like behavior after the first nicotine injection of the session day, a reaction not seen in the no-escalation group with the lowest dose of nicotine. Nicotine is known to evoke motor excitement including straub tail, tremors and convulsive responses [46–49]. We thus evaluated nicotine-induced behavioral excitement after the first daily nicotine injection using a four point-ranked score (see Material and Methods) [40]. The majority (roughly 75%) of the rats exhibited convulsive-like responses during the first days of self-administration at 240 µg/kg/injection (Fig. 2a), suggesting that nicotine has important negative-side effects. However, the incidence (Chi $X^2(n = 20,\ dl = 14) = 76.66$; $p < 0.001$; Fig. 2a) as well as the intensity ($F(14,266) = 7.81$; $p < 0.001$; Fig. 2b) of these convulsions decreased over repeated sessions presumably because of the development of a tolerance to nicotine-induced convulsive responses. These changes were, however, not correlated to escalated levels of nicotine intake ($r = -0.29$; NS). Importantly, despite these convulsions, rats not only continued to self-administer the high dose of nicotine, but were also even faster to initiate drug use at session onset in comparison to controls (1st injection latency: $F(44,1628) = 2.56$; $p < 0.001$; Fig. 2c and $F(2,78) = 4.79$; $p < 0.01$; Fig. 2d). Thus, apparently, the convulsions induced by the high dose of nicotine were not aversive, an interpretation further supported by the observation within the escalation group that rats with the more severe convulsive-like responses were also the faster to initiate drug intake ($r = -0.42$; $p < 0.05$, data not shown).

Escalation of nicotine alters vmPFC basal neuronal activity and its activity during ongoing nicotine self-administration

We next assessed changes in vmPFC neuronal activity during escalation of nicotine intake using in vivo electrophysiological recordings in another group of rats ($n = 10$; Fig. 3a). Tonic vmPFC neuronal activity was measured (i) after overnight abstinence during a 30 min pre-drug period before onset of nicotine self-administration (i.e., Before SA) and (ii) during the 3 h nicotine self-administration session (i.e., During SA). In the no-escalation group vmPFC neuronal activity before SA decreased rapidly after the initial sessions of nicotine intake ($F(2,102) = 3.47$; $p < 0.05$; Hab vs Initial SA, $p < 0.05$; Fig. 3b) and remained at the same level until the end of the procedure (Initial vs Final SA, NS; Fig. 3b), indicating that repeated nicotine self-administration inhibited basal vmPFC neuronal activity. In contrast in the escalation group, though an initial decrease in basal activity at the lowest dose of nicotine, vmPFC basal activity after nicotine escalation increased above pre-escalation levels ($F(2,132) = 4.48$; $p < 0.05$; Hab vs Initial SA, $p < 0.05$; Initial SA vs Final SA, $p < 0.01$; Fig. 3b) and above no-escalation activity levels observed at the end of the procedure ($F(1,76) = 4.91$; $p < 0.05$; Fig. 3b), suggesting a compensatory increase of initially depressed vmPFC neuronal activity after nicotine escalation.

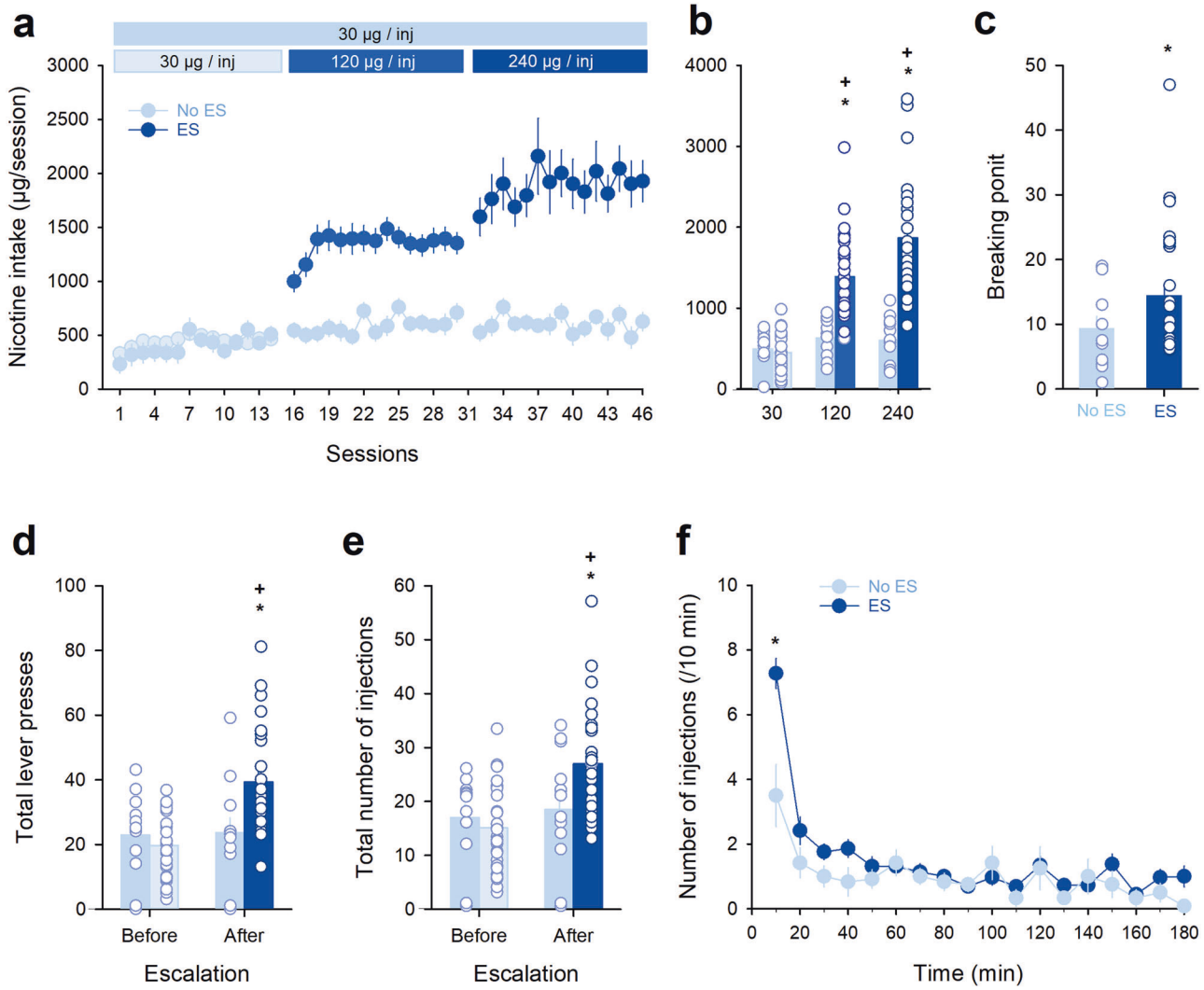

Fig. 1 Nicotine intake escaladed with increasing doses of nicotine. a Schematic diagram of the three stages nicotine escalation procedure (*top*). Mean (±SEM) nicotine intake during the 3 h session is plotted as a function of days for rats in the no-escalation group (No ES, light blue, $n = 12$) that has access to the unit dose of 30 µg/kg/injection, and rats in the escalation group (ES, blue gradient, $n = 27$) that had access to increasing doses of nicotine (30 µg, 120 µg and 240 µg/kg/injection) (*bottom*). **b** Average nicotine intake (mean ± SEM) during the last 3 days at each nicotine dose for the No ES and ES groups. **c** Mean (±SEM) breaking point reached at the end of the procedure for the No ES and ES groups (**d**, **e**) Mean (±SEM) number of lever presses (**d**) and nicotine injections (**e**) before and after escalation during a test session with the unit dose of nicotine 30 µg/kg/injection. **f** Mean number (±SEM) of nicotine injections per 10 min in the No ES and ES groups during the test session with the unit dose of nicotine 30 µg/kg/injection. *$p < 0.001$, different from the lowest dose of 30 µg/kg/injection. $^{+}p < 0.001$, different from the No ES group.

In addition to these basal neuronal adaptations, ongoing nicotine self-administration also directly affected vmPFC neuronal activity. It decreased overall firing rate activity during the entire 3 h nicotine self-administration session (i.e., During SA) compared to pre-dug baseline period (i.e., Before SA) in both groups ($F(1,67) = 12.4$; $p < 0.001$ and $F(1,88) = 74.9$; $p < 0.001$ for no-escalation and escalation groups, respectively; Fig. 3b, c), indicating that nicotine had inhibitory effects on vmPFC neuronal activity. However, while this inhibitory effect remained constant during repeated nicotine self-administration in the no-escalation group ($F(1,67) = 1.13$; NS; Fig. 3b), it intensified with repeated high doses of nicotine in the escalation group ($F(1,88) = 29.07$; $p < 0.001$; Initial SA Before vs During SA, *NS*; Final SA Before vs During SA, $p < 0.01$; Fig. 3b). Consistent with this, there was significantly more neurons showing a sustained decrease in firing rate during the self-administration session relative to the pre-drug baseline period in the escalation than in the no-escalation group (escalation 80% vs no-escalation 44%; $Z = 3.37$; $p < 0.001$; Fig. 3d), indicating stronger and more inhibitory neuronal responses in escalated than in non-escalated rats. Importantly, nicotine intake restored post-escalation changes in vmPFC neuronal activity as it brought the activity back to pre-escalation (Initial SA during SA vs Final SA during SA; NS; Fig. 3b) and no-escalation levels ($F(1,76) = 13.50$; $p < 0.001$; No ES Final SA vs ES Final SA; NS; Fig. 3b). Further quantification of the difference in firing activity before vs during SA (i.e., Δ overall firing activity) revealed that escalated rats with higher level of nicotine intake than non-escalated rats ($F(1,4) = 41.8$; $p < 0.01$; Fig. 3e) exhibited a higher vmPFC Δ overall firing activity ($F(1,4) = 10.8$; $p < 0.05$; Fig. 3f). This was further confirmed by looking at the long-duration changes in firing time-locked to nicotine self-injections (Fig. 3g, h). For most of the neurons it consisted of a change in firing rate, typically a decrease, within 1 or 2 min after the reinforced lever press followed by a progressive reversal of that change (Fig. 3g). Importantly, there were two times more neurons exhibiting long-duration decreases in firing after nicotine injection in the

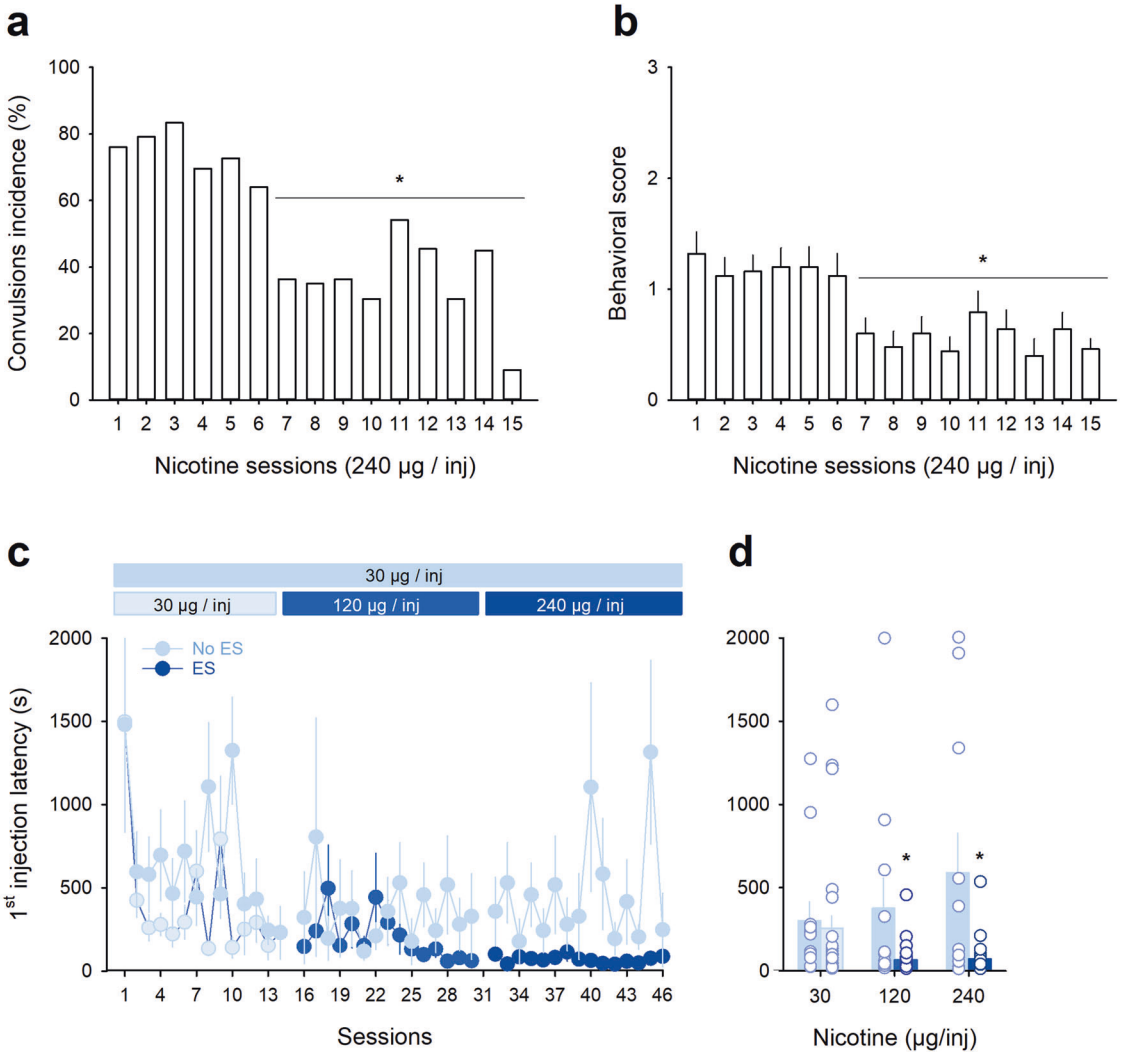

Fig. 2 Nicotine escalation induced convulsive-like behavior. a Incidence and (**b**) intensity (as revealed by the behavioral score) of the convulsive responses induced after the 1st injection of nicotine at 240 µg/kg/injection in the escalation group are plotted as a function of days. *$p < 0.01$, different from the 1st day. **c** Mean (±SEM) latency to initiate nicotine self-administration as a function of days and (**d**) averaged during the last 3 days at each nicotine dose for the No ES and ES groups. *$p < 0.01$, different from the lowest dose of 30 µg/kg/injection.

escalation than in the no-escalation group (47% and 78% for no-escalation and escalation groups respectively, $Z = 3.04$; $p < 0.01$; Fig. 3h). Together, these results suggest that nicotine intake induced larger vmPFC hypoactivity after nicotine escalation, and that this inhibitory effect can restore post-escalation changes in vmPFC neuronal activity.

Restoring basal vmPFC neuronal activity with in vivo optogenetic inhibition of vmPFC pyramidal neurons decreases nicotine self-administration in escalated rats

Based on these observations, we hypothesized that rats will escalate their drug intake in order to inhibit their vmPFC activity and thus to restore their basal vmPFC neuronal activity. If so, then mimicking the restoring effects of nicotine using in vivo optogenetic inhibition of vmPFC neurons during ongoing nicotine self-administration should decrease nicotine intake in escalated rats through a hedonic allostasis mechanism. To test this hypothesis, an adeno-associated virus targeting the pyramidal neurons and encoding the archaerhodopsin (CaMKII-ArChT3.0-eYFP) was bilaterally injected into the vmPFC with bilateral implantation of chronic optic fibers in the vmPFC (see Material and Methods; Fig. 4a). Efficiency of optical stimulation in inhibiting vmPFC pyramidal neurons activity was first controlled in an independent group of rats injected with ArChT3.0 ($n = 3$) in which vmPFC pyramidal neurons activity was strongly reduced by light application ($F(2,22) = 14.2$; $p < 0.001$; Fig. 4b). We next tested whether decreasing vmPFC pyramidal neurons activity could alter nicotine self-administration in two separates 1 h nicotine self-administration sessions (no light and light conditions; see Material and Methods) at the lowest dose of 30 µg/kg/injection. As expected from above, at this dose, rats in the escalation group pressed more (training condition effect: $F(1,22) = 23.28$; $p < 0.001$; Fig. 4c) and self-administered more nicotine than those in the no-escalation group ($F(1,22) = 15.31$; $p < 0.010$; Fig. 4d). Importantly, vmPFC optogenetic inhibition (250 ms pulse width at 1 Hz) during the 10 min pre-drug period before the onset of nicotine self-administration strongly decreased the total number of lever presses (training condition × virus type × light condition interaction: $F(1,22) = 4.66$; $p < 0.05$; ArChT-ES-No light vs ArChT-ES-Light, $p < 0.01$; ArChT-No ES-No light vs ArChT-No ES-Light, NS; Fig. 4c) and the total number of nicotine injections selectively in the escalation group injected with the archaerhodopsin but not in the no-escalation group (training condition × virus type × light condition interaction: $F(1,22) = 4.17$; $p < 0.05$; ArChT-ES-No light vs

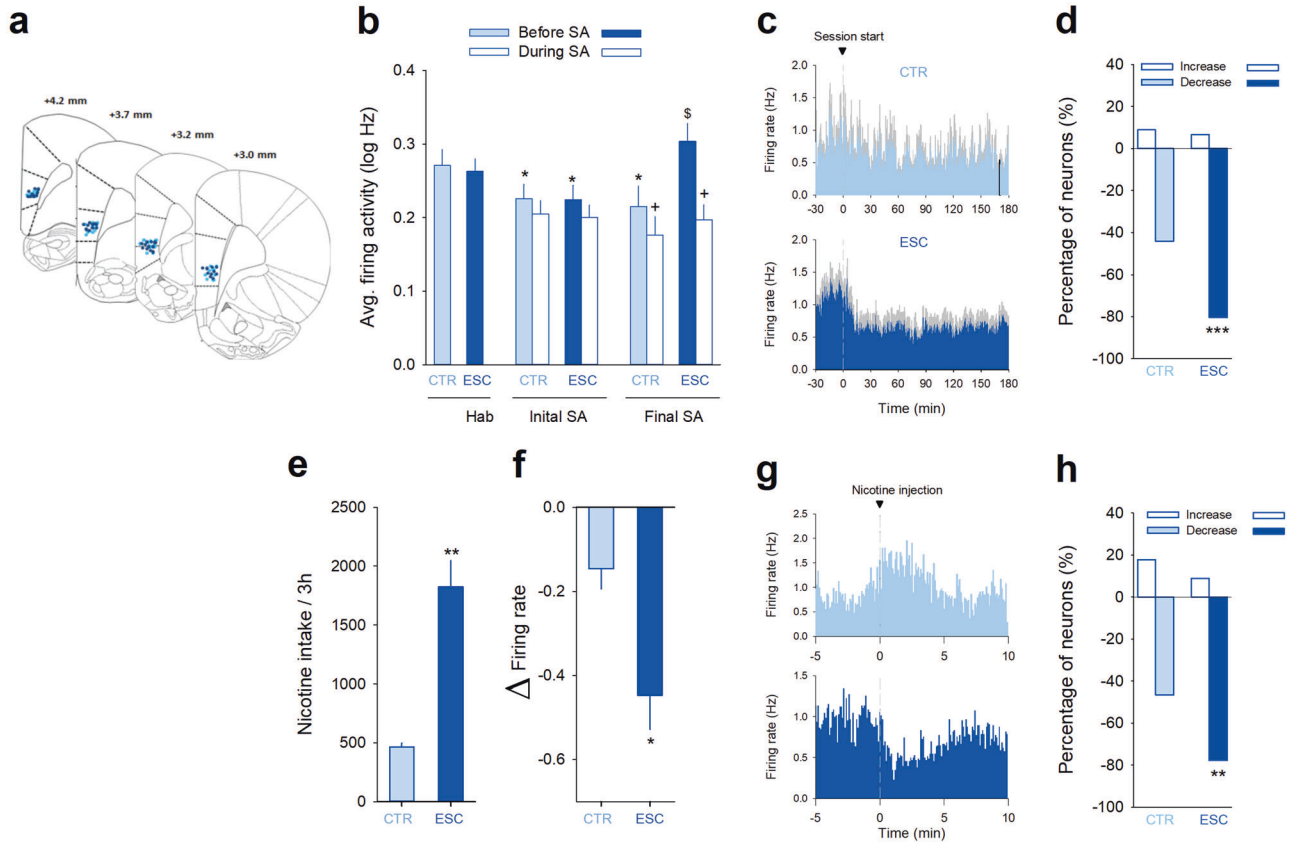

Fig. 3 Changes in vmPFC neuronal activity during nicotine intake escalation. a Schematic of the location of individual wire tips within the vmPFC. The numbers indicate millimeters anterior to bregma. **b** Mean (±SEM) tonic firing rates (log Hz) of vmPFC neurons measured 30 min period before (Before SA) and during the 3 h nicotine self-administration (During SA) across recording days in the no-escalation (No ES, $n = 5$) and the escalation (ES, $n = 5$) groups. Hab = habituation day; Initial SA = initial phase after 15 days of nicotine self-administration; and Final SA = final phase after 45 days of nicotine self-administration. *$p < 0.05$, different from Hab. $^{\$}p < 0.01$, different from Initial SA. $^{+}p < 0.01$, difference between Before SA vs During SA. **c** Average firing rate (Hz) of vmPFC neurons measured 30 min period before and during the 3 h nicotine self-administration during final SA is plotted as a function of time in the No ES and the ES groups. Time 0 on the abscissa corresponds to the start of the self-administration session. **d** Percentage of vmPFC neurons exhibiting a sustained increase (*white bars*) or decrease (*hatched bars*) in firing rate during the self-administration session relative to the pre-drug baseline period in the No ES and the ES groups. ***$p < 0.001$, different from No ES. Mean (±SEM) (**e**) nicotine intake and (**f**) difference in firing activity before vs during SA (Δ firing activity) in the No ES (*light blue*) and the ES (*dark blue*) groups during final SA. *$p < 0.05$ and **$p < 0.01$ different from No ES. **g** Examples of long-duration change in firing showing an increase *(light blue)* or a decrease (*dark blue*) in firing. Each perievent histogram shows the average firing rate of a single neuron 5 min before and 10 min after nicotine injection. Time 0 on the abscissa corresponds to the nicotine injection. **h** Percentage of vmPFC neurons exhibiting a long-duration increase (*white bars*) or decrease (*hatched bars*) in firing rate after nicotine injection in the No ES (*light blue*) and the ES (*dark blue*) groups. **$p < 0.01$, different from No ES.

ArChT-ES-Light, $p < 0.05$; ArChT-No ES-No light vs ArChT-No ES-Light, *NS*; Fig. 4d). This effect was particularly pronounced during the first 10 min of nicotine self-administration corresponding to the drug-loading behavioral period for both the number of lever presses ($F(5,55) = 3.10$; $p < 0.01$; ArChT-ES-No light 10 min vs ArChT-ES-Light 10 min, $p < 0.01$; Fig. 4e) and the number of nicotine injections ($F_(5,55) = 3.65$; $p < 0.05$; ArChT-ES-No light 10 min vs ArChT-ES-Light 10 min, $p < 0.01$; Fig. 4f). Similar results were obtained when the vmPFC optogenetic inhibition was performed during the first 10 min of nicotine self-administration ($F_{(5,55)} = 2.42$; $p < 0.05$; ES-No light 10 min vs ES-Light 10 min, $p < 0.01$ and $F_{(5,55)} = 2.42$; $p < 0.05$; ES-No light 10 min vs ES-Light 10 min, $p < 0.01$; data not shown). Importantly, this was specific to nicotine escalation as vmPFC optogenetic inhibition had no effect on responding for sucrose ($F(1,7) = 2.59$; *NS*; Fig. 5b) in a separate group of rats trained to lever press under a FR1 schedule of reinforcement. In addition, after initial training under a FR1, the same rats were tested with a FR3 to further increase the response rate, but despite this, their behavior remained unchanged during vmPFC optogenetic inhibition ($F(1,7) = 0.04$; *NS*; Fig. 5c).

DISCUSSION

Access to unusually high doses of nicotine precipitated a rapid and robust escalation of nicotine intake in rats that progressed despite the emergence of convulsion-like behavior in the majority of rats. In addition, once established escalated levels of nicotine intake were associated with an increased motivation to take the drug and an increased daily drug-loading behavior at session onset, and were defended when the unit dose of nicotine was decreased. These phenomena were previously observed with other drugs after escalation ([7]; for a systematic review, see [50]) and are thought to represent a behavioral signature of an increased motivation for higher states of intoxication. Escalation of nicotine intake had been difficult to obtain previously [21, 22, 51, 52], except under specific access conditions, such as, for instance, intermittent access to extended periods of nicotine self-administration, separated by long periods of forced abstinence (i.e., 21–23 h sessions every 24–72 h) [19, 20]. As shown here, previous difficulties in inducing robust escalation of nicotine intake are also probably due, at least partly, to the use of low unit doses of nicotine, though other factors are also involved.

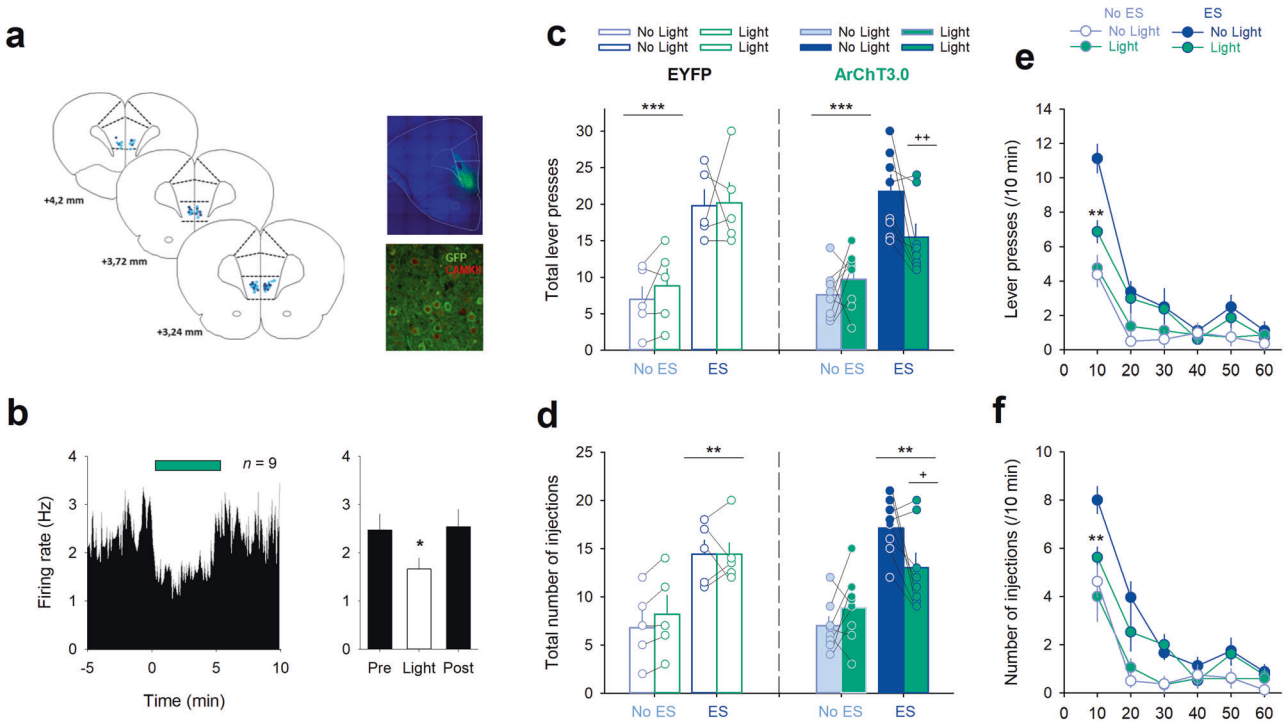

Fig. 4 In vivo optogenetic inhibition of vmPFC decreased nicotine self-administration. a (*Left*) Schematic of the location of individual optic fiber tips within the vmPFC. The numbers indicate millimeters anterior to bregma. (*Right*) Representative images of coronal sections showing the virus expression and fibers implants in the vmPFC (*top*) and immunostaining of eYFP (green) expression in vmPFC pyramidal neurons (red, CAMKII) (*bottom*). **b** (*Left*) Average firing frequency (Hz) of light-evoked responses in vmPFC neurons expressing ArchT3.0. (*Right*) Mean (±SEM) firing rate prior (Pre), during (Light) and after (Post) stimulation application in vmPFC neurons expressing ArchT3.0. *$p < 0.01$, as compared to pre- and post-light. **c**, **d** Mean (±SEM) total number of (**c**) lever presses and (**d**) nicotine injections during the 1 h nicotine self-administration session at 30 µg/kg/injection in the no-escalation (No ES) and the escalation groups (ES) injected either with eYFP ($n = 5$ for each group) or ArChT3.0 ($n = 8$ for each group) under light or no-light conditions. ***$p < 0.001$ and **$p < 0.01$, different from the No ES group. $^{++}p < 0.01$ and $^{+}p < 0.05$, different from No Light. **e**, **f** (**e**) Response time course and (**f**) and injection time course are plotted as a function of time in the no-escalation (No ES) and the escalation group (ES) injected with ArChT3.0 ($n = 8$ for each group). **$p < 0.01$, different from No Light.

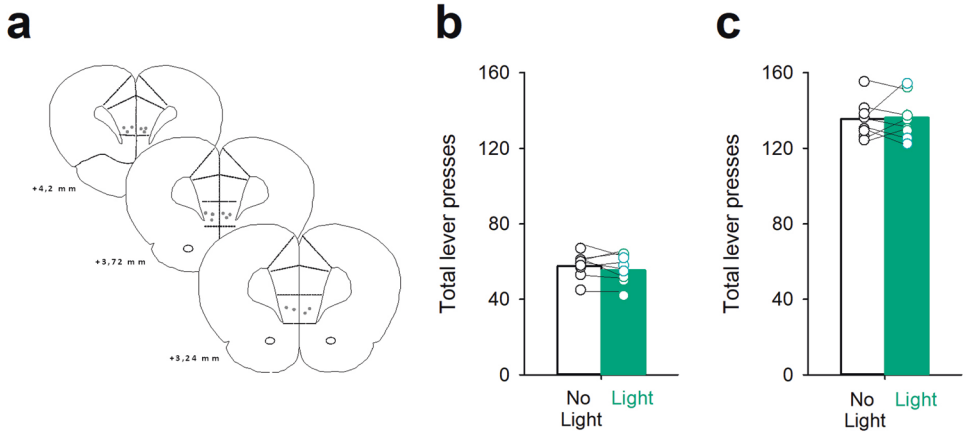

Fig. 5 In vivo optogenetic inhibition of vmPFC had no effect on sucrose self-administration. a Schematic of the location of individual optic fiber tips within the vmPFC. The numbers indicate millimeters anterior to bregma. **b**, **c** Mean (±SEM) total number of lever presses during (**b**) FR1 and (**c**) FR3 sucrose self-administration in rats injected with ArChT3.0 ($n = 8$).

Transition to nicotine addiction is thought to result from neuroadaptative changes that increase the need to continue using tobacco [53, 54]. Here we report that escalation of nicotine intake induced long-lasting changes in vmPFC basal neuronal activity (i.e., activity recorded before onset of nicotine self-administration session). Specifically, after escalation of nicotine intake, basal vmPFC neuronal activity increased above pre-escalation and no-escalation activity levels. Similar alterations in mPFC neurons firing rate have been previously observed with repeated psychostimulant treatments [55, 56] or after withdrawal from psychostimulants self-administration [57, 58]. Together, these results suggest that the post-escalation basal changes in neuronal activity could indicate the instauration of a new allostatic state in the vmPFC during drug intake escalation, a neurophysiological adaptation that might be common to escalation of intake across different drugs.

Ongoing nicotine self-administration also altered vmPFC neuronal activity. Despite an heterogeneous population response, there was a net decrease of vmPFC neuronal activity during the entire self-administration session compared to pre-dug baseline period, as well as during few minutes after the nicotine self-injection. Importantly, greater and more sustained inhibitory responses were observed in rats with escalated levels of nicotine intake, suggesting that this nicotine-induced vmPFC hypoactivity may contribute to the motivation to take the drug during nicotine intake escalation. As such, we hypothesized that rats will escalate their nicotine intake in order to inhibit their vmPFC neuronal activity in an attempt to compensate for basal changes in vmPFC neuronal activity, and that interventions simulating these restoring effects of nicotine on vmPFC neuronal activity should thus decrease escalated levels of nicotine use. Consistent with our hypothesis, the optogenetic inhibition of vmPFC pyramidal neurons before or at the onset of nicotine self-administration strongly decreased nicotine self-administration selectively in rats that had escalated their nicotine intake, thus suggesting that part of the motivation for using nicotine during escalation might be to restore post-escalation changes in vmPFC basal neuronal activity. However, these data do not prove that rats self-administer nicotine in order to inhibit elevated vmPFC basal activity, and future research is thus needed to more directly test this hypothesis. Importantly, this effect was specific to nicotine intake escalation as it was not observed in the no-escalation group or in rats trained to respond at high rate for sucrose. Though conducting the converse experiment with optical excitation of vmPFC neurons may add to this conclusion, this would require being able to reproduce reliably with optogenetic stimulation the complex changes in vmPFC neuronal activity that were observed after nicotine intake escalation—a delicate task that is beyond the scope of the present study (see also, below).

The finding that vmPFC optogenetic inhibition decreased nicotine intake is consistent with previous report showing that vmPFC inhibition reduced methamphetamine and heroin seeking after extinction or cocaine seeking after abstinence [29, 35, 36, 59]. However, opposite behavioral outcomes have also been reported [44, 60], suggesting a more complex role for the vmPFC in drug-taking and seeking behaviors. Interestingly, growing evidence indicates that separate, but intermingled, neural ensembles within vmPFC selectively encode different and even opposite behaviors [31, 32, 35]. Though we did not determine which vmPFC neuronal ensembles are involved, the heterogeneous vmPFC neuronal responses observed here during nicotine self-administration is consistent with this conceptualization.

Finally, it is important to stress that our interpretation is not inconsistent with other possible interpretations of nicotine intake escalation. For instance, abstinence from nicotine is also known to produce robust affective withdrawal symptoms, including increased anxiety-like behavior [20, 61, 62], symptoms that have been associated with increased probability for escalating smoking behavior [6, 63]. Moreover, vmPFC has been implicated in anxiety regulatory processes [64, 65] and vmPFC lesion or inactivation reduced anxiety-like behaviors [66]. Thus, it is also possible that rats during overnight abstinence exhibited an increased level of anxiety [67, 68] that they seek to relieve through increased nicotine use resumption. In addition, post-escalation changes in basal vmPFC neuronal activity can result from altered glutamatergic [69, 70] and/or cholinergic signaling [71]. Indeed, withdrawal from nicotine self-administration down-regulates metabotropic glutamate receptors (mGluRs) function in the mPFC [72, 73], and NMDA or mGluR5s antagonists increase mPFC neurons firing [55, 74, 75]. Moreover, chronic nicotine administration also leads to a rapid desensitization of nicotinic acetylcholine receptors (nAChRs) in the mPFC that are known to regulate the neuronal activity of mPFC pyramidal neurons [76–78].

To sum up, our findings add to growing evidence that drug intake escalation induces changes in vmPFC neuronal function and suggest that interventions aimed at correcting these changes may prove useful in the treatment of addiction.

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AUTHOR CONTRIBUTIONS

KG and SHA designed research and experiments; CVM, KG, and MA performed behavioral experiments and associated data analysis; GRF and KG performed in vivo electrophysiology and optogenetic experiments, and associated data analysis; GRF performed immunohistochemistry; KG and SHA wrote the paper.

FUNDING

This work was supported by the French Research Council (CNRS), the Université de Bordeaux, and the French National Agency (ANR- 15-CE37-0008-01; KG). The authors have nothing to disclose.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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