Title: Serotonin neurons in the median raphe nucleus bidirectionally regulate somatic signs of nicotine withdrawal in mice

Article type: Research Article

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ABSTRACT

In chronic smokers, nicotine withdrawal symptoms during tobacco cessation can lead to smoking relapse. In rodent models, chronic exposure to nicotine elicited physical dependence, whereas acute antagonism of nicotinic acetylcholine receptors (nAChRs) immediately precipitated withdrawal symptoms. Although the central serotonergic system plays an important role in nicotine withdrawal, the exact serotonergic raphe nuclei regulating these symptoms remain unknown. We used transgenic mice expressing archaerhodopsinTP009 or channelrhodopsin-2[C128S] exclusively in the central serotonergic neurons to selectively manipulate serotonergic neurons in each raphe nucleus. Nicotine withdrawal symptoms were precipitated by an acute injection of mecamylamine, a nonspecific nAChR antagonist, following chronic nicotine consumption. Somatic signs were used as measures of nicotine withdrawal symptoms. Acute mecamylamine administration significantly increased ptosis occurrence in nicotine-drinking mice compared with that in control-drinking mice. Optogenetic inhibition of the serotonergic neurons in the median raphe nucleus (MRN), but not of those in the dorsal raphe nucleus (DRN), mimicked the symptoms observed during mecamylamine-precipitated nicotine withdrawal even in nicotine-naïve mice following the administration of acute mecamylamine injection. Optogenetic activation of the serotonergic neurons in the MRN nearly abolished the occurrence of ptosis in nicotine-drinking mice. The serotonergic neurons in the MRN, but not those in the DRN, are necessary for the occurrence of somatic signs, a nicotine withdrawal symptom, and the activation of these neurons may act as a potential therapeutic strategy for preventing the somatic manifestations of nicotine withdrawal.

Keywords: tobacco; smoking; 5-HT; optogenetics; cholinergic

1. Introduction

In chronic smokers, abrupt tobacco cessation causes various withdrawal symptoms such as decreased arousal and irritability [1]. Several lines of evidence have indicated that the central serotonergic system plays a key role in nicotine withdrawal. For example, through human and animal studies, chronic nicotine administration has been shown to reduce serotonin levels in the hippocampus [2,3]. We previously reported that the precursor of serotonin, 5-hydroxytryptophan, relieves nicotine withdrawal symptoms in rats [4]. In recent rodent studies, it has been shown that the interpeduncular nucleus is involved in nicotine withdrawal symptoms [5] and that the nucleus projects to serotonergic nuclei, e.g., the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) [6]. However, the precise nucleus (DRN or MRN) responsible for nicotine withdrawal symptoms remains unknown to date.

To address this issue, we administered mice nicotine-containing water for over 6 weeks. Nicotine withdrawal symptoms were precipitated by a nonspecific nicotinic acetylcholine receptor antagonist, mecamylamine, following chronic nicotine consumption [5]. The precipitation procedure enables a within-subject design if required. In the present study, somatic signs such as ptosis were used as measures of nicotine withdrawal symptoms, and they were measured using visual observation (Experiment 1).

After replicating the results of a previous study [5], we examined whether serotonergic inhibition could induce these symptoms in nicotine-naïve mice. To this end, we developed transgenic mice expressing archaerhodopsinTP009 (ArchT) [7], a yellow light-driven neuronal silencer, exclusively in the central serotonergic neurons. We previously confirmed the validity of these mice using histological and electrophysiological methods [8]. We applied yellow light to the DRN or MRN of nicotine-naïve mice that received acute mecamylamine administration and observed their symptoms (Experiment 2).

Furthermore, we determined whether serotonergic activation could attenuate the symptoms

in mice that consumed nicotine-containing water for over 6 weeks. To selectively activate serotonergic neurons, we used previously established transgenic mice (*Tph2*-tTA::tetO-ChR2(C128S)-EYFP bi-transgenic mice) [9,10] expressing channelrhodopsin-2 step-function-type variant (ChR2[C128S]) only in the central serotonergic neurons. Serotonergic neurons expressing this ChR2 variant are activated by blue light and remain activated for a few minutes until the application of yellow light [9] (Experiment 3).

2. Materials and methods

2.1. General information of animals

Adult male and female mice (C57BL/6N or transgenic mice with a C57BL/6N background and aged >56 days at the beginning of nicotine administration or surgery) were used. Approximately equal numbers of male and female mice were used. The mice were housed in groups prior to starting drug administration or surgery. The animal rooms were maintained under an alternating light–dark cycle (light from 7 p.m. to 7 a.m.) at approximately 25 °C. All tests were performed during the dark period. The treatment of animals complied with the Guidelines for the care and use of Laboratory Animals of the Animal Research Committee of Hokkaido University.

2.2. Experiment 1: Nicotine withdrawal symptoms in wild type mice

2.2.1. Animals

A total of 48 adult male and female wild-type mice were used: 13 mice consumed a control solution and received saline injections, 11 mice consumed a control solution and received mecamylamine injections, 12 mice consumed a nicotine solution and received saline injections, and 12 mice consumed a nicotine solution and received mecamylamine injections.

2.2.2. Drug and nicotine administration

All drugs were purchased from Sigma-Aldrich (St. Louis, USA). To prepare the nicotine and control solutions

not included because these symptoms were not observed. Observers were blinded to the experimental conditions.

2.2.4. Statistical analysis

Behavioral parameters were analyzed using a two-factor ANOVA with nicotine/control solutions and saline/mecamylamine injection as the between-subject factors. When there was a significant drinking–injection interaction, a one-factor ANOVA was conducted after the two-factor ANOVA. The alpha level was set at 0.05 (two-tailed) for all comparisons. All statistical procedures were conducted using SPSS version 23.0 (IBM, NY, USA).

2.3. Experiment 2: Nicotine withdrawal-like symptoms in transgenic mice expressing ArchT in serotonergic neurons

2.3.1. Animals

We created animals expressing ArchT in the central serotonergic neurons by crossing a tetracycline operator (tetO)-ArchT–enhanced yellow fluorescent protein (EYFP) BAC transgenic mouse line (RRID:IMSR_RBRC05842) with a *Tph2*-tTA BAC transgenic mouse line (RRID:IMSR_RBRC05846). The production of the tetO-ArchT BAC transgenic mice and *Tph2*-tTA BAC transgenic mice has been described previously [7,9,10]. These mice were backcrossed to the C57BL/6N strain for more than six generations. A total of 33 mice were used: 6 mice received no light to the MRN, 10 received yellow light to the MRN, 7 received no light to the DRN, and 10 received yellow light to the DRN.

2.3.2. Surgical procedure

The mice were anesthetized with isoflurane (4% for induction and 1% for maintenance) and fixed in a stereotaxic frame (Narishige, Tokyo, Japan). For the application of light to the DRN or MRN, an optic fiber with a mirror tip at 45° (MA45; Doric Lenses, Quebec, Canada) was implanted with coordinates 4.5 mm posterior to the bregma, 1.0 mm lateral to the midline, and

2.3 (DRN) or 3.7 (MRN) mm ventral to the dura [15]. After surgery, ointments containing antibiotics and steroids (Dolmycin, Zeria Pharmaceutical Co., Ltd., Tokyo, Japan; Kenalog, Bristol-Myers Squibb, NewYork, NY, USA) were applied to the wounds. A piece of jelly containing carprofen (MediGel,Clear H_2O, Portland, ME, USA) was placed in the home cage, and the mice were housed individually and allowed to recover for 7 days prior to behavioral experiments.

2.3.3. In vivo light illumination

For light applications to the DRN or MRN, yellow (575 nm) light was generated by a SPECTRA 2-LCR-XA light engine (Lumencor, Beaverton, OR, USA), and the light intensity at the fiber tip was maintained at approximately 1 mW/mm^2. A fiber optic rotary joint (Doric Lenses, Quebec, Canada) was used for unrestricted *in vivo* illumination, and the light was controlled via TTL pulses driven by a stimulator (Nihon Kohden, Tokyo, Japan).

2.3.4. Somatic signs

The mice received an intraperitoneal injection of mecamylamine (3 mg/kg) and were immediately placed in a clear plastic observation chamber (22 × 15 × 13 cm) without bedding. Counting of somatic signs was performed as described in Experiment 1.

2.3.5. Verification of optical fiber placements

After completion of the experiments, the optical fiber placements were verified via visual inspection under a microscope. After intracardial perfusion with 4% paraformaldehyde in PBS (pH 7.2), the mice brains were post-fixed overnight and placed in 0.1 M PB containing 20% sucrose. Further, 50- m-thick coronal sections were cut on a cryostat and mounted onto slides. After drying, the sections were stained with toluidine blue, and the cannula placements were verified under a microscope according to the atlas [15]. Data of the mice with incorrect placements were excluded from the analysis.

2.3.6. Statistical analysis

Each somatic sign in each group (DRN or MRN) was separately analyzed using Student's *t*-test with yellow light or no light as the between-subject factor.

2.4. Experiment 3: Nicotine withdrawal symptoms in transgenic mice expressing ChR2 in serotonergic neurons

2.4.1. Animals

We generated animals expressing ChR2 in the central serotonergic neurons by crossing a tetO-ChR2(C128S)–EYFP knock-in mouse line (RRID:IMSR_RBRC05454) with a *Tph2*-tTA BAC transgenic mouse line. The development of these mice has been described previously [7,9,10]. These mice were backcrossed to the C57BL/6N strain for more than 10 generations. A total of eight mice were used who received yellow and blue light to the MRN as described later.

2.4.2. Surgical procedure

The mice were anesthetized with isoflurane and fixed in a stereotaxic frame (Narishige) as described in Experiment 2. For light applications to the MRN, an optic fiber with a mirror tip at 45° (MA45; Doric Lenses) was implanted with coordinates 4.5 mm posterior to the bregma, 1.0 mm lateral to the midline, and 3.7 mm ventral to the dura [15]. After surgery, the mice were housed individually and allowed to recover for 7 days prior to starting nicotine administration. The rest of the procedures was the same as that for Experiment 2.

2.4.3. Drug and nicotine administration

Nicotine solutions were prepared and administered as described in Experiment 1. After the 6-week administration of nicotine solutions, 10 ml/kg saline or mecamylamine hydrochloride (3 mg/kg) was intraperitoneally injected into the mice prior to the start of the behavioral experiment.

2.4.4. In vivo light illumination

For light applications to the MRN, blue (475 nm) or yellow (575 nm) light was generated by a

SPECTRA 2-LCR-XA light engine (Lumencor). The rest of the experimental setup was the same as that for Experiment 2. We used yellow light as a control because yellow light itself does not induce any change in neural activity [9]. Blue or yellow lights were applied to the MRN (once per minute, 500-ms duration) during somatic sign observation. We previously demonstrated that blue light pulses using this procedure increased serotonin release [9].

2.4.5. Somatic signs

The mice received an intraperitoneal injection of mecamylamine (3 mg/kg) and were immediately placed into a clear plastic observation chamber (22 × 15 × 13 cm) without bedding. We counted somatic signs as described in Experiment 1. In this experiment, however, two somatic sign observation tests were conducted on different days for each mouse: once with blue and once with yellow light illumination. The order of light illumination was counterbalanced across animals. Each test was conducted at an interval of more than a day.

2.4.6. Verification of optical fiber placements

After completion of the experiments, optical fiber placements were verified under a microscope as for Experiment 2.

2.4.7. Statistical analysis

Each somatic sign was separately analyzed using paired *t*-test.

3. Results

3.1. Experiment 1: Nicotine withdrawal symptoms in wild type mice

Of the somatic signs, ptosis occurrence was increased by acute mecamylamine injection only in the mice that consumed nicotine solution for 6 weeks (Figure 1B; drinking–injection interaction, $F_{1,44} = 47.895$, $P < 0.001$; followed by a one-way ANOVA analysis for each injection: $F_{1,23} = 0.92$, $P = 0.347$ in saline-injected mice [n = 25]; $F_{1,21} = 20.187$, $P < 0.001$ in mecamylamine-injected mice [n = 23]). Other somatic signs were not altered by acute

mecamylamine injection, whether or not the mice consumed nicotine solutions (Figure 1B; drinking–injection interaction, $Fs_{1,44} < 1.322$, *NS*; drinking, $Fs_{1,44} < 1.780$, *NS*; injection, $Fs_{1,44} < 3.221$, *NS*). The total number of miscellaneous, less frequent signs was also increased by acute mecamylamine injection only in the mice that consumed nicotine solutions (Figure 1B; drinking–injection interaction, $F_{1,44} = 6.672$, $P = 0.0132$; followed by a one-way ANOVA analysis for each injection: $F_{1,23} = 0.446$, $P = 0.511$ in saline-injected mice [n = 25]; $F_{1,21} = 7.105$, $P = 0.012$ in mecamylamine-injected mice [n = 23]).

3.2. Experiment 2: Nicotine withdrawal symptoms in transgenic mice expressing ArchT in serotonergic neurons

Behavioral experiments showed that ptosis occurrence was increased by acute mecamylamine injection when yellow light was applied to the MRN, but not to the DRN (Figure 2D, F; DRN: $t_{15} = 0.268$, $P = 0.792$ [n = 17]; MRN: $t_{14} = 2.728$, $P = 0.016$ [n = 16]). Other somatic signs were not altered by acute mecamylamine injection when the light was applied to the DRN or MRN (Figure 2D, F; DRN: $ts_{15} < 1.494$, *NS* [n = 17]; MRN: $ts_{14} < 2.014$, *NS* [n = 16]).

3.3. Experiment 3: Nicotine withdrawal symptoms in transgenic mice expressing ChR2 in serotonergic neurons

Next, we examined whether serotonergic activation in the MRN could attenuate somatic signs in mice that consumed nicotine-containing water for over 6 weeks. We found that mecamylamine-precipitated ptosis was almost completely reversed by blue light application to the MRN (Figure 3D $t_7 = 4.246$, $P = 0.004$ [n = 8]). However, other somatic signs were not altered by blue light application to the MRN (Figure 3D; $ts_7 < 1.871$, *NS* [n = 8]).

4. DISCUSSION

We replicated some, but not all, nicotine withdrawal symptoms in mice. We observed

mecamylamine-precipitated ptosis in mice that consumed nicotine solutions, consistent with the findings of previous studies [5,16]. However, we failed to observe an increase in terms of other somatic signs such as scratching, rearing, and body shaking (Figure 1), inconsistent with the findings of previous studies [5,17]. It appears that the type of somatic signs exhibited differs among species [18-20] and laboratories [5,12,16,17,21], although the reasons for these differences have not yet been determined. The way in which withdrawal signs are expressed possibly differs among individuals because the total number of miscellaneous, less frequent signs was increased by acute mecamylamine injection, although the number of each of these signs was not significantly changed.

We found that the combination of serotonergic inhibition in the MRN and acute mecamylamine injection elicited somatic signs (Figure 2). The observation that nicotine withdrawal symptoms can be induced even in nicotine-naïve mice was remarkable. However, it raises the question of how this is possible. Given that cholinergic innervation of the interpeduncular nucleus is involved in the inhibition of GABAergic projection neurons and cholinergic disinhibition induces nicotine withdrawal symptoms (see Figure 6 in [5]) and that GABA neurons in the interpeduncular nucleus project toward the DRN and MRN [6], nicotine withdrawal/nicotinic acetylcholine receptor antagonists could disinhibit GABAergic projection neurons in the interpeduncular nucleus and inhibit the raphe nuclei, thereby inducing nicotine withdrawal symptoms. Thus, the optogenetic manipulation used in Experiment 2 and the acute blockade of nicotinic acetylcholine receptors could converge on serotonergic inhibition. Whether they have a synergetic effect on serotonergic activity should be addressed in future studies.

It is unlikely that yellow light itself or only serotonergic inhibition without mecamylamine induces ptosis for two reasons. First, the application of yellow light to the DRN with mecamylamine in ArchT-expressing mice did not elicit ptosis, indicating that the yellow light

itself has no effect on somatic signs (Figure 2). Second, our preliminary results (n = 3) showed that this light application to the MRN without mecamylamine did not elicit ptosis (Supplementary Table 1), implying that serotonergic inhibition without mecamylamine cannot elicit ptosis.

We showed that the MRN, and not the DRN, is responsible for the occurrence of somatic signs of nicotine withdrawal symptoms. A recent study demonstrated that decreased connectivity of the median raphe nucleus and hippocampus is linked to the development of nicotine withdrawal symptoms [22]. Optogenetic activation or inhibition of serotonergic neurons in the MRN might increase or decrease the connectivity, thereby mitigating or eliciting nicotine withdrawal symptoms, respectively. Although it is unknown how this connectivity regulates withdrawal symptoms, our results support this hypothesis.

The DRN is possibly involved in other withdrawal symptoms, such as depression, although the present study did not identify any relationships between serotonergic function in the DRN and somatic signs. The functions of the DRN have previously been associated with drug withdrawal symptoms [23,24] and with depression [25-27]. However, this issue is beyond of the scope of this study.

Overall, our results suggest that the MRN plays a pivotal role in inducing somatic signs, a symptom of nicotine withdrawal. Any manipulations activating serotonergic neurons in the MRN are potentially therapeutical for some symptoms of nicotine withdrawal. However, the question of whether the present findings could be extended to other symptoms of nicotine withdrawal should be addressed in future studies aimed at developing more efficient stop-smoking aids.

Acknowledgments

This work was supported by the JSPS KAKENHI grants (numbers: JP25713043, 18K07545,

and JP16K15552) awarded to Y.O. and a Grant from the Smoking Research Foundation (URL: http://www.srf.or.jp/english/index.html) awarded to M.Y. We would like to thank Tomoko Furukawa and Aki Tanimori for their help in breeding the transgenic mice, and thank Enago (www.enago.jp) for the English language review.

ADDITIONAL INFORMATION

Supplemental information accompanies this paper.

Disclosure: The authors declare no competing interests.

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Figure legends

Fig. 1. Acute mecamylamine injection-precipitated nicotine withdrawal symptoms in nicotine-drinking mice

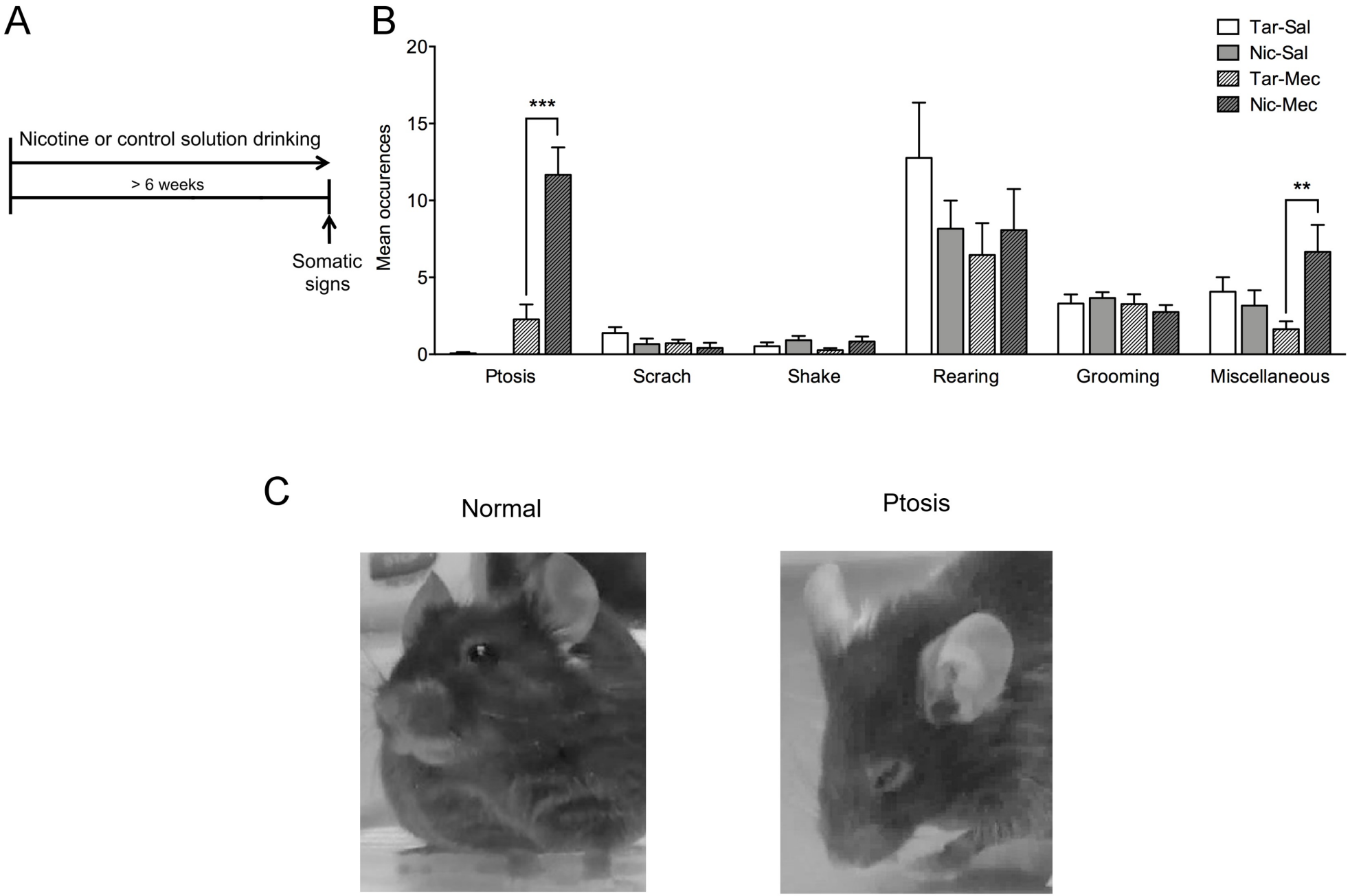
(A) Schematic representation of the experimental time course for mecamylamine-precipitated nicotine withdrawal symptoms. **(B)** Acute mecamylamine injection significantly increased ptosis occurrence in nicotine-drinking mice. **(C)** An example of ptosis. The eye was almost closed compared to normal condition. $^{*}P < 0.05$, n = 11–13 for each group. The bars represent mean values, whereas the lines represent SEM values.

Fig. 2. Effects of optogenetic inhibition of the serotonergic neurons in the median or dorsal raphe nucleus combined with acute mecamylamine injection on somatic signs

(A) Mice expressing central serotonergic neuron-specific ArchT were obtained by crossing a tetO-ArchT BAC transgenic mouse line with a Tph2-tTA line. **(B)** Schematic representation of the experimental time course for nicotine-naïve mice. **(C)** Optical fiber placements. We considered the optical fibers placed in the area surrounded by gray dot lines were correct. One optical fiber placement was incorrect (gray fiber tip). Two optical fiber placements were considered incorrect because of too anterior placement, but not indicated in the figure. **(D)** Optogenetic inhibition of the dorsal raphe serotonergic neurons did not alter ptosis occurrence but significantly induced other somatic signs. n = 6–10 for each group. **(E)** Optical fiber placements. We considered the optical fibers placed in the area surrounded by gray dot lines were correct. One optical fiber placement was considered incorrect because of too anterior placement, but not indicated in the figure. **(F)** Optogenetic inhibition of the median raphe serotonergic neurons significantly increased ptosis occurrence but did not alter other somatic signs. $^{*}P < 0.05$, n = 7–10 for each group. The bars represent mean values, and the lines represent SEM values.

Fig. 3. Effects of optogenetic activation of the serotonergic neurons in the median raphe nucleus on mecamylamine-precipitated somatic signs in nicotine-drinking mice

(A) Mice expressing central serotonergic neuron-specific ChR2[C128S] were obtained by crossing a tetO-ChR2[C128S] knock-in mouse line with a Tph2-tTA line. **(B)** Schematic representation of the experimental time course for mecamylamine-precipitated nicotine withdrawal symptoms. **(C)** Optical fiber placements. We considered the optical fibers placed in the area surrounded by gray dot lines were correct. **(D)** Optogenetic activation of the median raphe serotonergic neurons significantly reduced ptosis occurrence but did not affect other somatic signs. $^{*}P < 0.05$, n = 8. The bars represent mean values, and the lines represent SEM values.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Nicotine or control solution drinking
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Ptosis
Scrach
Shake
Rearing
Grooming
Miscellaneous
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Normal
Ptosis

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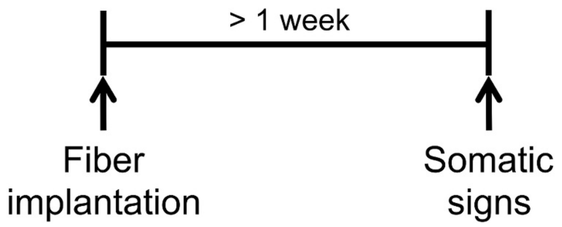
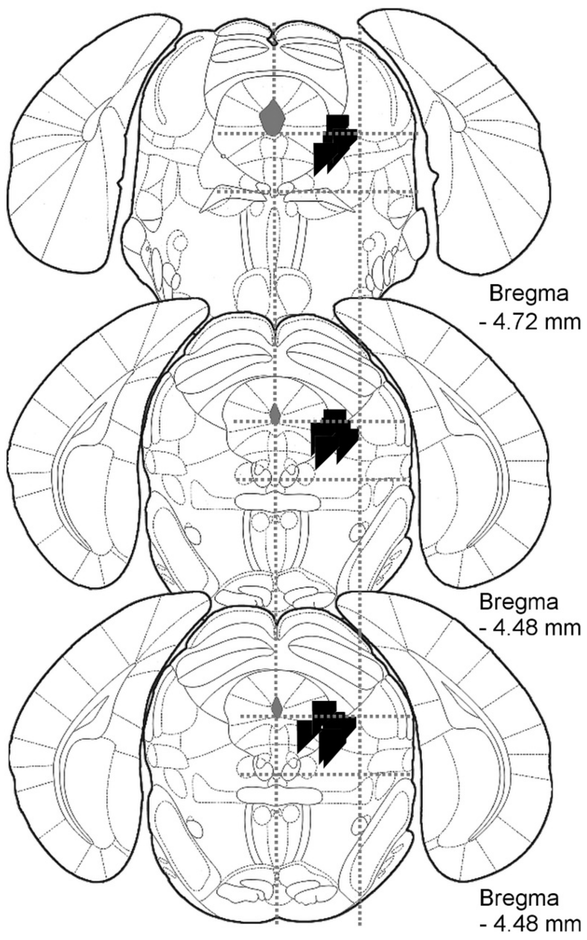
Tph2-tTA
tetO-ArchT
Bi-transgenic
Serotonergic neuron-
specific ArchT expression

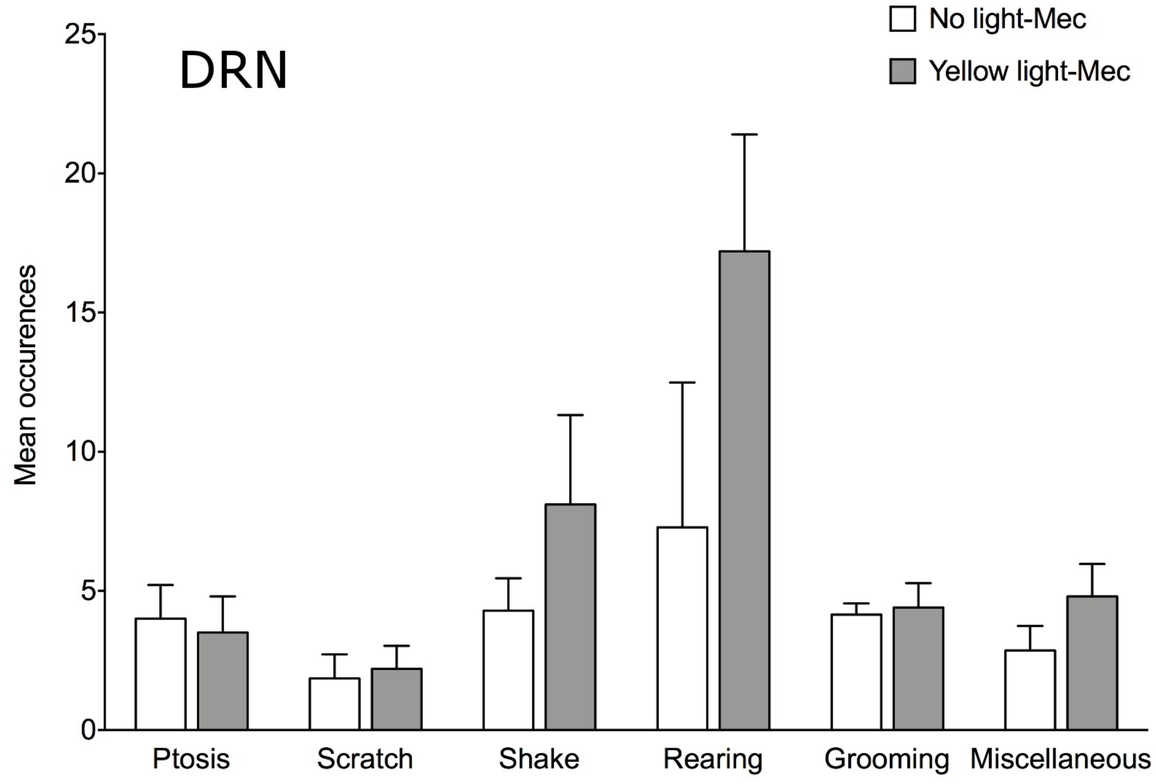
B


> 1 week
Fiber
implantation
Somatic
signs

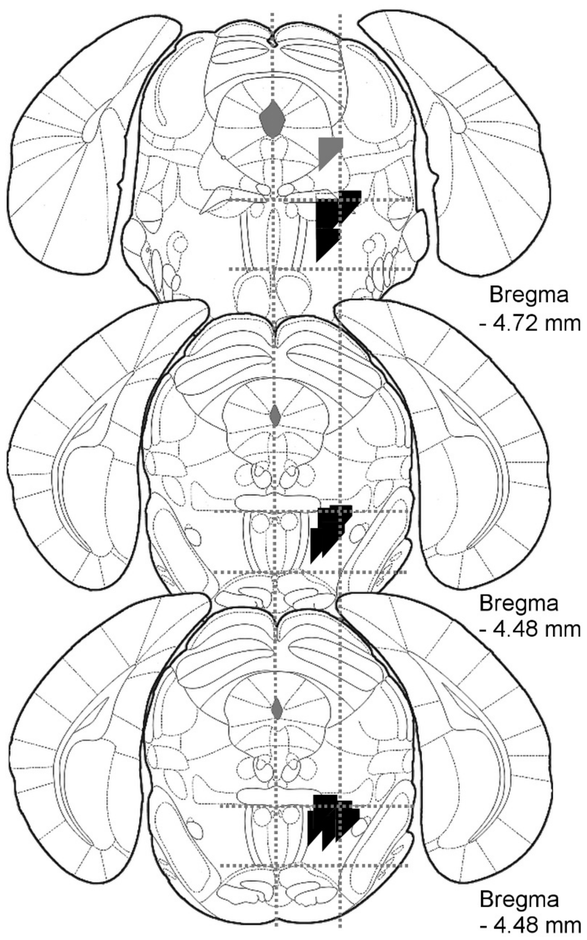
C


Bregma
- 4.72 mm
Bregma
- 4.48 mm
Bregma
- 4.48 mm

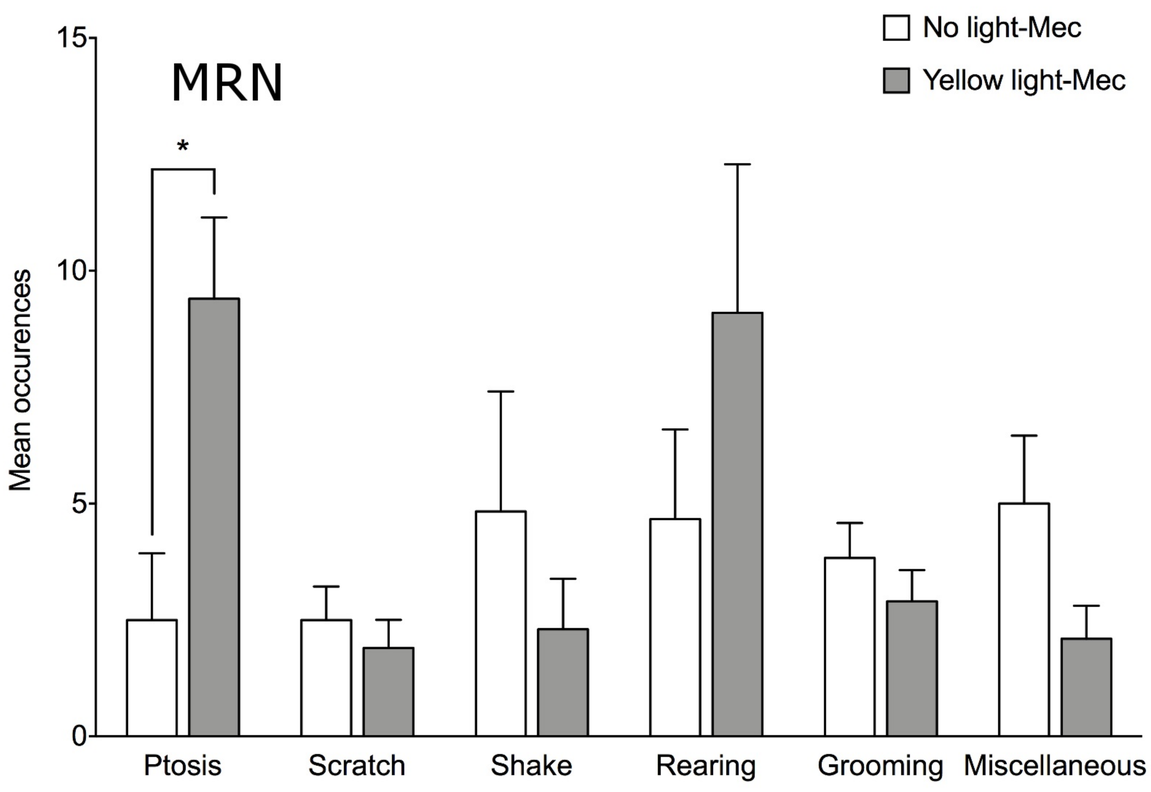
D


DRN
No light-Mec
Yellow light-Mec
Mean occurences
25
20
15
10
5
0
Ptosis
Scratch
Shake
Rearing
Grooming
Miscellaneous

E


Bregma
- 4.72 mm
Bregma
- 4.48 mm
Bregma
- 4.48 mm

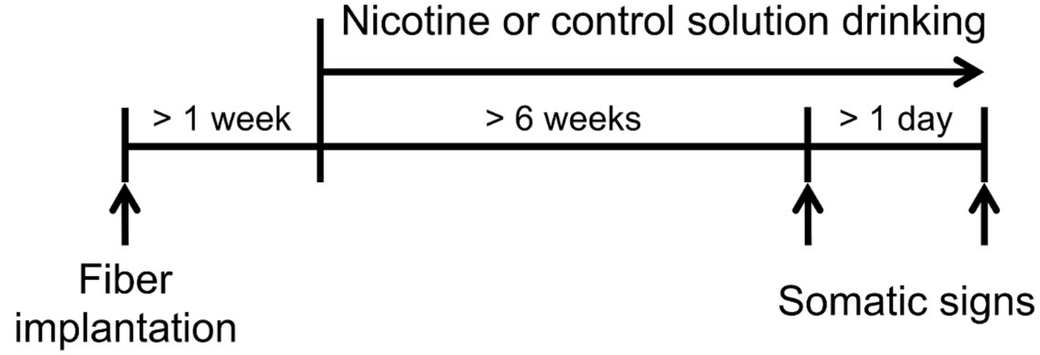
F


MRN
No light-Mec
Yellow light-Mec
*
Mean occurences
15
10
5
0
Ptosis
Scratch
Shake
Rearing
Grooming
Miscellaneous

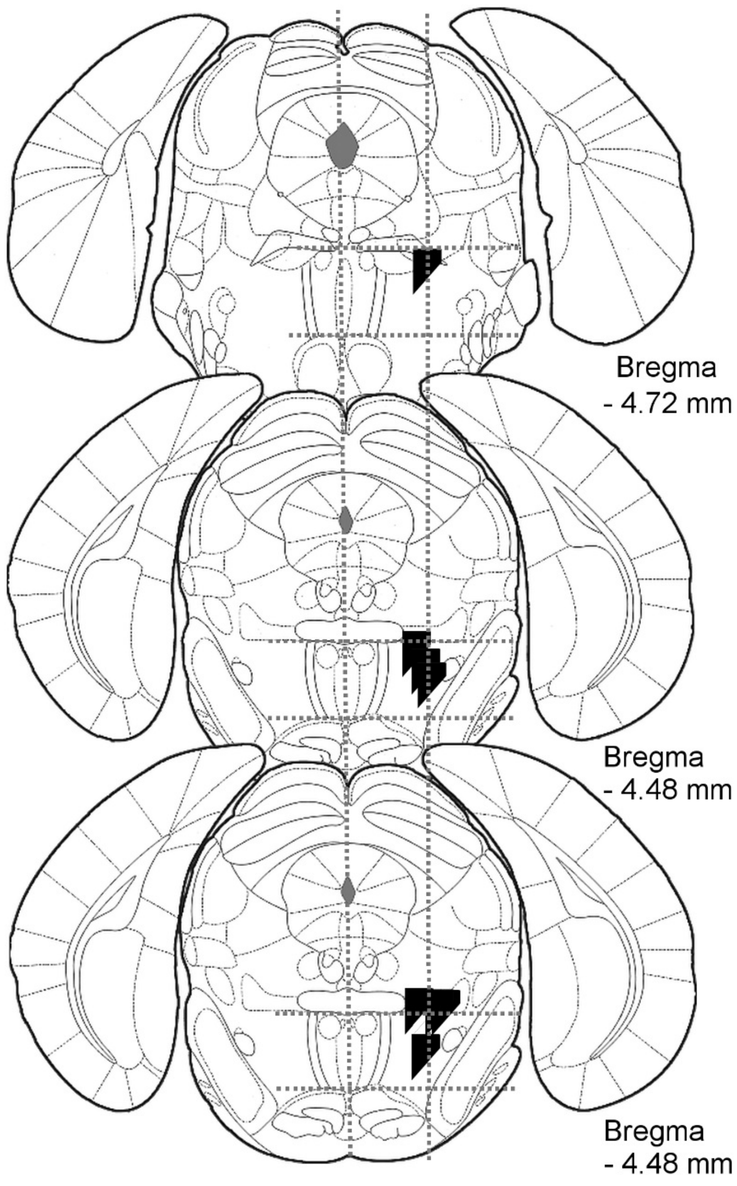
A


Tph2-tTA
tetO-ChR2[C128S]
Bi-transgenic
Serotonergic neuron-specific
ChR2[C128S] expression

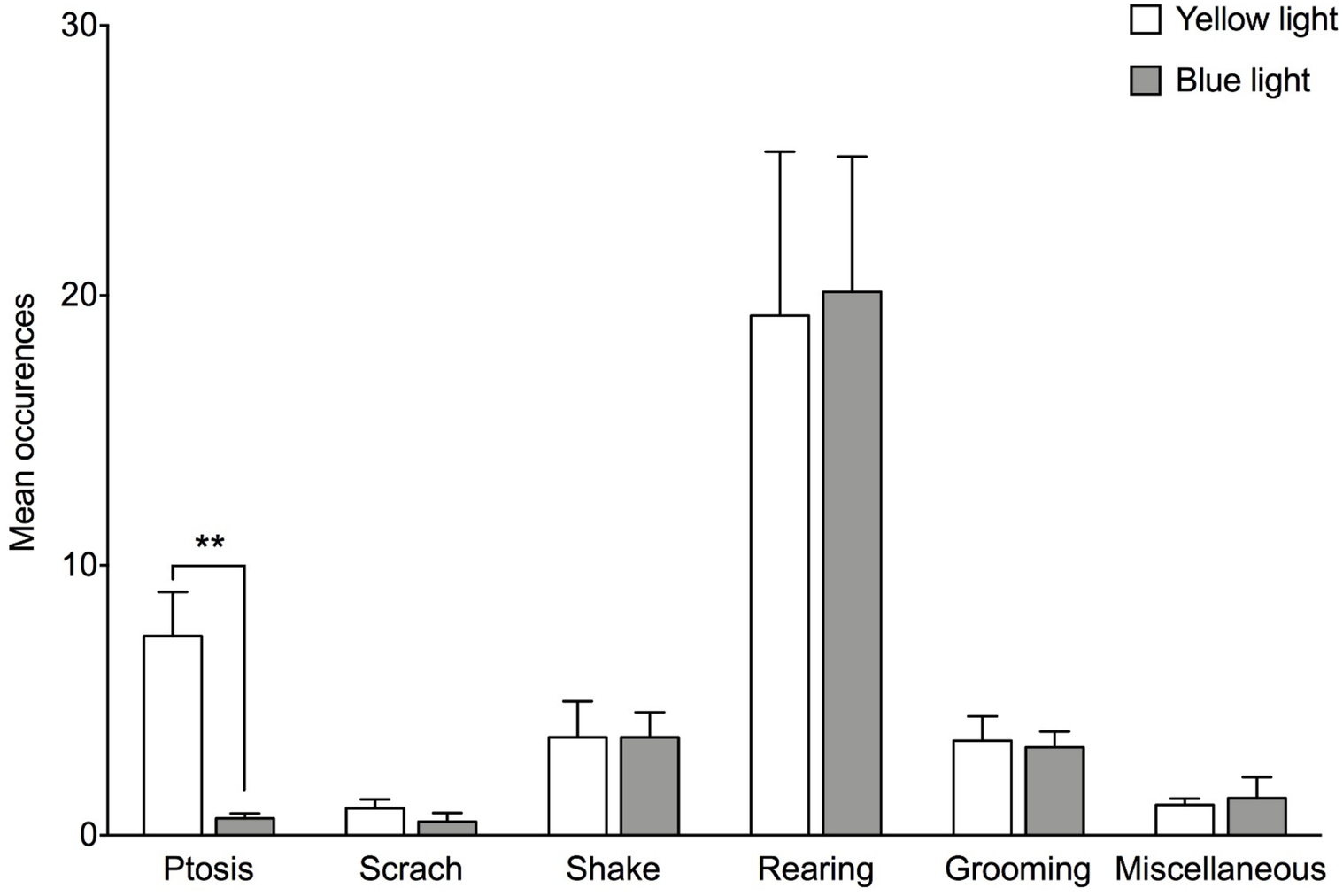
B


Nicotine or control solution drinking
> 1 week
> 6 weeks
> 1 day
Fiber
implantation
Somatic signs

C


Bregma
- 4.72 mm
Bregma
- 4.48 mm
Bregma
- 4.48 mm

D


Yellow light
Blue light
Mean occurences
30
20
10
0
**
Ptosis
Scrach
Shake
Rearing
Grooming
Miscellaneous