



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

3 **Article type:** Research Article

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1 **ABSTRACT**

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

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

23

1 **1. Introduction**

2 In chronic smokers, abrupt tobacco cessation causes various withdrawal symptoms such as
3 decreased arousal and irritability [1]. Several lines of evidence have indicated that the central
4 serotonergic system plays a key role in nicotine withdrawal. For example, through human and
5 animal studies, chronic nicotine administration has been shown to reduce serotonin levels in the
6 hippocampus [2,3]. We previously reported that the precursor of serotonin,
7 5-hydroxytryptophan, relieves nicotine withdrawal symptoms in rats [4]. In recent rodent
8 studies, it has been shown that the interpeduncular nucleus is involved in nicotine withdrawal
9 symptoms [5] and that the nucleus projects to serotonergic nuclei, e.g., the dorsal raphe nucleus
10 (DRN) and median raphe nucleus (MRN) [6]. However, the precise nucleus (DRN or MRN)
11 responsible for nicotine withdrawal symptoms remains unknown to date.

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

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

25 Furthermore, we determined whether serotonergic activation could attenuate the symptoms

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

7

8 **2. Materials and methods**

9 *2.1. General information of animals*

10 Adult male and female mice (C57BL/6N or transgenic mice with a C57BL/6N background and
11 aged >56 days at the beginning of nicotine administration or surgery) were used.

12 Approximately equal numbers of male and female mice were used. The mice were housed in
13 groups prior to starting drug administration or surgery. The animal rooms were maintained
14 under an alternating light–dark cycle (light from 7 p.m. to 7 a.m.) at approximately 25 °C. All
15 tests were performed during the dark period. The treatment of animals complied with the
16 Guidelines for the care and use of Laboratory Animals of the Animal Research Committee of
17 Hokkaido University.

18

19 *2.2. Experiment 1: Nicotine withdrawal symptoms in wild type mice*

20 *2.2.1. Animals*

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

25 *2.2.2. Drug and nicotine administration*

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

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

3 *2.2.4. Statistical analysis*

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

9

10 *2.3. Experiment 2: Nicotine withdrawal-like symptoms in transgenic mice expressing ArchT in* 11 *serotonergic neurons*

12 *2.3.1. Animals*

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

21 *2.3.2. Surgical procedure*

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

1 2.3 (DRN) or 3.7 (MRN) mm ventral to the dura [15]. After surgery, ointments containing
2 antibiotics and steroids (Dolmycin, Zeria Pharmaceutical Co., Ltd., Tokyo, Japan; Kenalog,
3 Bristol-Myers Squibb, New York, NY, USA) were applied to the wounds. A piece of jelly
4 containing carprofen (MediGel, Clear H₂O, Portland, ME, USA) was placed in the home cage,
5 and the mice were housed individually and allowed to recover for 7 days prior to behavioral
6 experiments.

7 *2.3.3. In vivo light illumination*

8 For light applications to the DRN or MRN, yellow (575 nm) light was generated by a
9 SPECTRA 2-LCR-XA light engine (Lumencor, Beaverton, OR, USA), and the light intensity at
10 the fiber tip was maintained at approximately 1 mW/mm². A fiber optic rotary joint (Doric
11 Lenses, Quebec, Canada) was used for unrestricted *in vivo* illumination, and the light was
12 controlled via TTL pulses driven by a stimulator (Nihon Kohden, Tokyo, Japan).

13 *2.3.4. Somatic signs*

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

17 *2.3.5. Verification of optical fiber placements*

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

25 *2.3.6. Statistical analysis*

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

3

4 *2.4. Experiment 3: Nicotine withdrawal symptoms in transgenic mice expressing ChR2 in* 5 *serotonergic neurons*

6 *2.4.1. Animals*

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

12 *2.4.2. Surgical procedure*

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

19 *2.4.3. Drug and nicotine administration*

20 Nicotine solutions were prepared and administered as described in Experiment 1. After the
21 6-week administration of nicotine solutions, 10 ml/kg saline or mecamylamine hydrochloride
22 (3 mg/kg) was intraperitoneally injected into the mice prior to the start of the behavioral
23 experiment.

24 *2.4.4. In vivo light illumination*

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

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

6 *2.4.5. Somatic signs*

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

13 *2.4.6. Verification of optical fiber placements*

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

16 *2.4.7. Statistical analysis*

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

18

19 **3. Results**

20 *3.1. Experiment 1: Nicotine withdrawal symptoms in wild type mice*

21 Of the somatic signs, ptosis occurrence was increased by acute mecamlamine injection only in
22 the mice that consumed nicotine solution for 6 weeks (Figure 1B; drinking–injection interaction,
23 $F_{1,44} = 47.895$, $P < 0.001$; followed by a one-way ANOVA analysis for each injection: $F_{1,23} =$
24 0.92 , $P = 0.347$ in saline-injected mice [$n = 25$]; $F_{1,21} = 20.187$, $P < 0.001$ in
25 mecamlamine-injected mice [$n = 23$]). Other somatic signs were not altered by acute

1 mecamylamine injection, whether or not the mice consumed nicotine solutions (Figure 1B;
2 drinking–injection interaction, $F_{S1,44} < 1.322$, *NS*; drinking, $F_{S1,44} < 1.780$, *NS*; injection, $F_{S1,44}$
3 < 3.221 , *NS*). The total number of miscellaneous, less frequent signs was also increased by
4 acute mecamylamine injection only in the mice that consumed nicotine solutions (Figure 1B;
5 drinking–injection interaction, $F_{1,44} = 6.672$, $P = 0.0132$; followed by a one-way ANOVA
6 analysis for each injection: $F_{1,23} = 0.446$, $P = 0.511$ in saline-injected mice [$n = 25$]; $F_{1,21} =$
7 7.105 , $P = 0.012$ in mecamylamine-injected mice [$n = 23$]).

8 *3.2. Experiment 2: Nicotine withdrawal symptoms in transgenic mice expressing ArchT in* 9 *serotonergic neurons*

10 Behavioral experiments showed that ptosis occurrence was increased by acute
11 mecamylamine injection when yellow light was applied to the MRN, but not to the DRN
12 (Figure 2D, F; DRN: $t_{15} = 0.268$, $P = 0.792$ [$n = 17$]; MRN: $t_{14} = 2.728$, $P = 0.016$ [$n = 16$]).
13 Other somatic signs were not altered by acute mecamylamine injection when the light was
14 applied to the DRN or MRN (Figure 2D, F; DRN: $t_{S15} < 1.494$, *NS* [$n = 17$]; MRN: $t_{S14} < 2.014$,
15 *NS* [$n = 16$]).

16 17 *3.3. Experiment 3: Nicotine withdrawal symptoms in transgenic mice expressing Chr2 in* 18 *serotonergic neurons*

19 Next, we examined whether serotonergic activation in the MRN could attenuate somatic
20 signs in mice that consumed nicotine-containing water for over 6 weeks. We found that
21 mecamylamine-precipitated ptosis was almost completely reversed by blue light application to
22 the MRN (Figure 3D $t_7 = 4.246$, $P = 0.004$ [$n = 8$]). However, other somatic signs were not
23 altered by blue light application to the MRN (Figure 3D; $t_{S7} < 1.871$, *NS* [$n = 8$]).

24

25 **4. DISCUSSION**

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

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

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

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

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

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

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

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

23

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5

6 **ADDITIONAL INFORMATION**

7 Supplemental information accompanies this paper.

8

9 **Disclosure:** The authors declare no competing interests.

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29

1 **Figure legends**

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

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

9

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

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

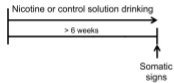
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2 **Fig. 3. Effects of optogenetic activation of the serotonergic neurons in the median raphe**
3 **nucleus on mecamylamine-precipitated somatic signs in nicotine-drinking mice**

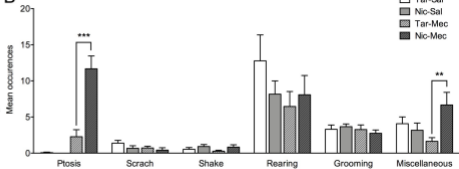
4 **(A)** Mice expressing central serotonergic neuron-specific ChR2[C128S] were obtained by
5 crossing a tetO-ChR2[C128S] knock-in mouse line with a Tph2-tTA line. **(B)** Schematic
6 representation of the experimental time course for mecamylamine-precipitated nicotine
7 withdrawal symptoms. **(C)**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

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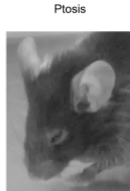
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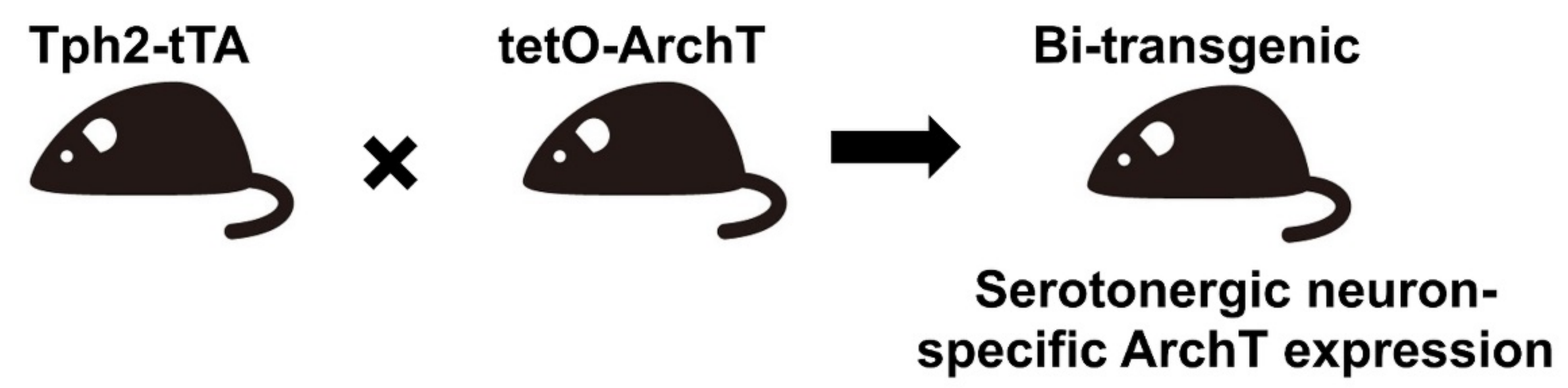
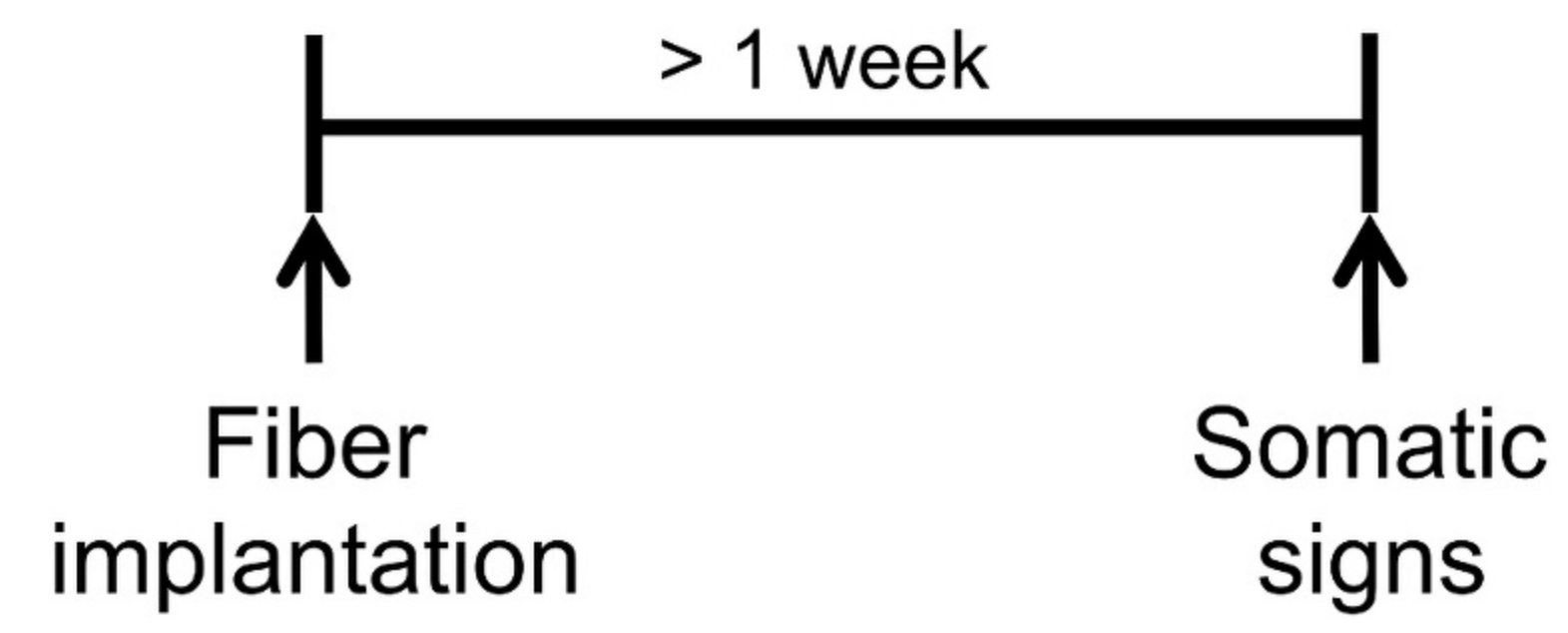
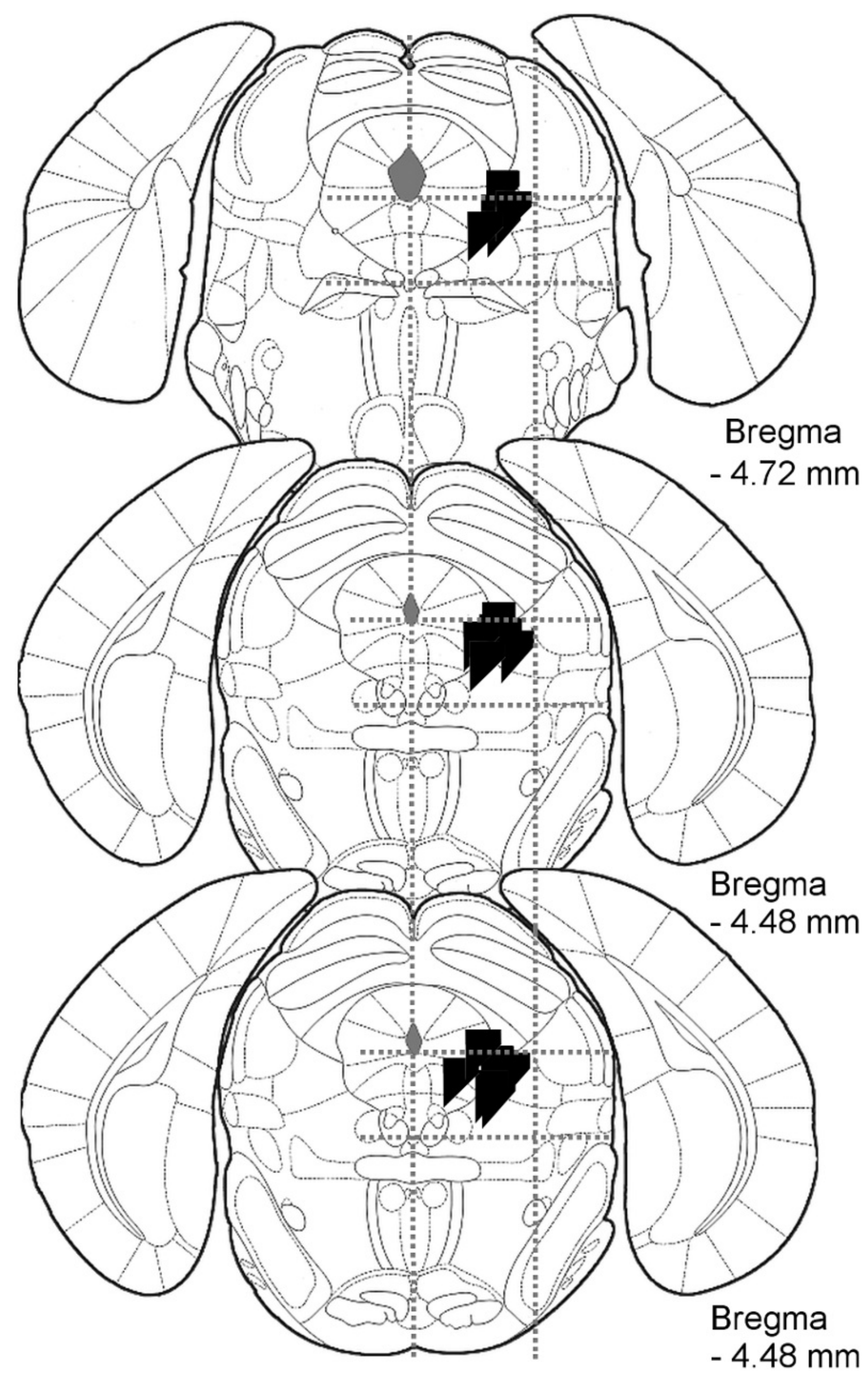
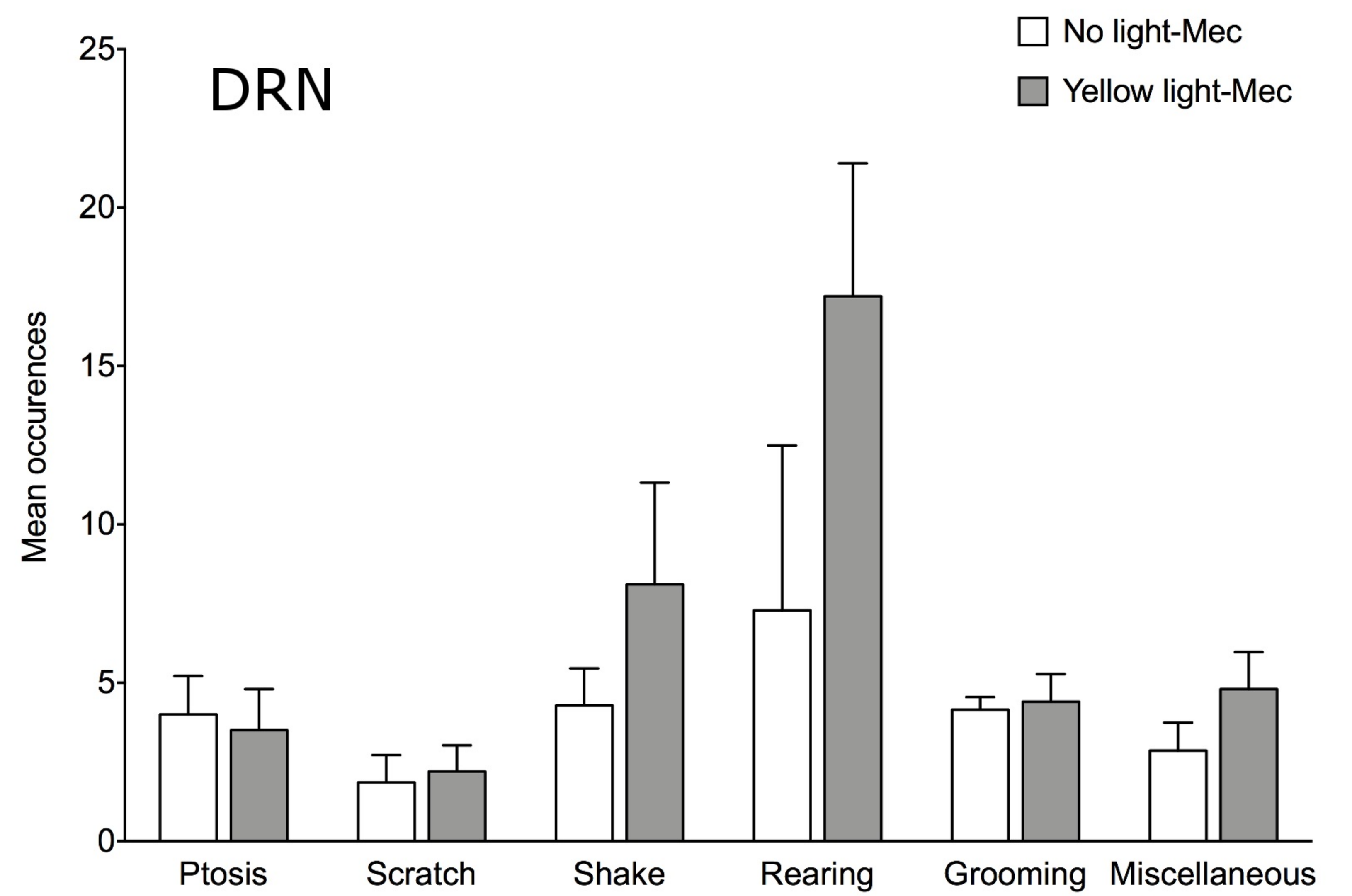
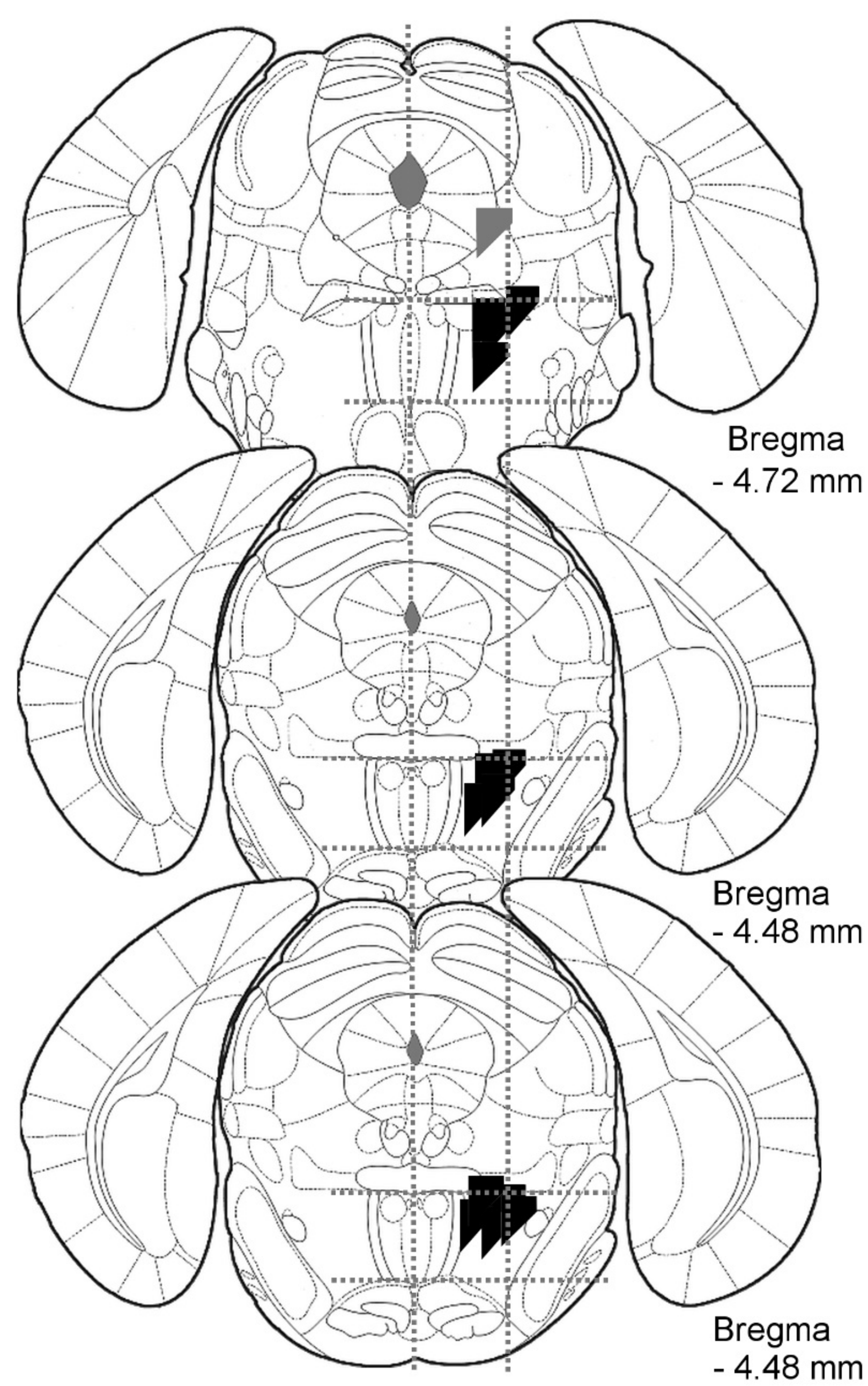


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