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Region-specific effects of copulation on dendritic spine morphology and gene expression related to spinogenesis in the medial preoptic nucleus of male rats

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ABSTRACT

The medial preoptic nucleus (MPN) plays an essential role in the control of male sexual behavior. In rats, the central part of the MPN (MPNc) contains a sexually dimorphic nucleus exhibiting male-biased morphological sex differences. Although it has been suggested that the MPNc of male rats functions to induce sexual arousal, the mechanisms by which male rats are sexually aroused to successfully achieve copulation are poorly understood. We recently showed that increased neuronal activity in the MPNc of male rats during copulation is higher at their first copulation compared with later copulations, indicating that a plastic change in excitatory synaptic transmission occurs with copulatory experience. In this study, we tested the hypothesis that changes to dendritic spines at structural and molecular levels occur following copulatory experience. First, we examined the effects of at least two copulations on the morphology of dendrites and spines in the MPNc and in the lateral and medial parts of the MPN (MPNlm) of male rats. In the MPNc, the total number of dendrites and their branches, and the surface area of dendrites were not significantly affected by copulation. However, the copulatory experience, specifically experience of ejaculation, significantly reduced the density of mushroom spines but not of filopodia, thin or stubby spines in the MPNc. In the MPNlm, the copulatory experience, specifically experience of ejaculation, significantly increased the surface area of dendrites, although there was no significant effect of copulation on spine density. Next, we measured the mRNA levels of genes encoding actin-binding proteins related to spinogenesis after male rats had copulated for their first and second times. Copulatory stimuli, especially stimuli from ejaculation, significantly reduced the mRNA levels of drebrin A and spinophilin in the MPNc but not in the MPNlm. These results indicate that copulatory experiences, especially experience of ejaculation, reduce spine density in the MPNc of male rats, which may result, in part, from downregulation of genes encoding actin-binding proteins.

1. Introduction

Appropriate modulation of sexual activity in sexually reproducing animals is important not only for species survival but also for better communication with conspecifics of the opposite sex. Adult males are intrinsically motivated to copulate, and thereby, males actively display sexual behavior when they encounter sexually attractive females. Additionally, it has been demonstrated that the motivation of male rats to copulate is reinforced by copulatory experience, reflecting a greater efficiency of sexual behavior for subsequent copulation opportunities (Bialy et al., 2000; Dewsbury, 1969; Lopez et al., 1999). Thus, sexual activity of males increases in response to successful copulation.

The neural systems controlling male sexual behavior in rodents are well described (Hull and Dominguez, 2015) with the medial preoptic nucleus (MPN) playing an important role in rodents and many species (Dominguez and Hull, 2005; Hull and Dominguez, 2015). Electrical stimulation of the MPN induces male sexual behavior in rats (Malsbury, 1971; Merari and Ginton, 1975). In contrast, lesions of the MPN disrupt sexual behavior in male rats (Arendash and Gorski, 1983; Hansen et al., 1982; Kondo et al., 1990). This and other accumulating evidence indicates that the MPN of male rats is a regulatory center that receives olfactory stimuli from sexually receptive female rats and somatosensory stimuli from their genitalia via the medial amygdala and the bed nucleus of the stria terminalis (BNST) (Hull and Dominguez, 2006; Veening and Coolen, 2014). The MPN then transmits neural information to lower brain stem regions, such as the ventral tegmental area,

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periaqueductal gray, and paragigantocellular nucleus, to control sexual behavior and ejaculation (Hull and Dominguez, 2006; Veening and Coolen, 2014). Thus, the MPN is a core part of the system regulating male sexual behavior.

The MPN is composed of three distinct subdivisions (Simerly, 1995). Cells in the lateral part of the MPN are sparsely distributed compared with other subdivisions, while the medial part of the MPN contains cells densely. The central part of the MPN (MPNc), which is embedded in the medial part of the MPN, is a compact and very cell-dense part. The MPN of rats and homologous regions in other species are sexually dimorphic (Woodson and Gorski, 1999). Specifically, the MPNc in rats is a sexually dimorphic nucleus that exhibits male-biased sex differences in volume and neuron number (Gorski et al., 1978, 1980). The neurochemical characteristics of the MPNc are different from the lateral and medial parts of the MPN (MPNlm). The MPNc contains many neurons expressing calbindin-D28 K and monooxygenase DBH-like 1, but the MPNlm contains few such neurons (Sickel and McCarthy, 2000; Tsuneoka et al., 2017). Neural connectivity of the MPNc also differs from that of the MPNlm (Simerly and Swanson, 1986, 1988). One of the differences in neural connectivity between the MPNc and MPNlm is a reciprocal connection to the BNST, which is involved in the control of male sexual behavior as well as the MPN (Claro et al., 1995). More MPNc neurons receives neuronal inputs from the BNST and project to the BNST than do MPNlm neurons (Simerly and Swanson, 1986, 1988). In humans, the interstitial nucleus of the anterior hypothalamus (INAH) is regarded as homologous with the MPNc (Allen et al., 1989; Swaab and Hofman, 1995), which may support the idea that the INAH of men functions similarly to the MPNc of male rats, although the physiological functions of these brain regions are not well understood.

As mentioned earlier, the MPN contributes significantly to the control of sexual behavior in male rats, although the physiological roles of the MPNc in male sexual behavior require further investigation. In sexually experienced male rats that have ejaculated at least twice, lesions of the MPNc have no significant effect on male sexual performance (Arendash and Gorski, 1983), while lesions in the MPNc induce a delay of sexual behavior in sexually naive male rats (De Jonge et al., 1989). We previously showed by analysis of c-Fos expression that the MPNc contains a neuronal population that activates during the first copulation and then silences after acquisition of their first copulatory experience (Yamaguchi et al., 2018). We also suggested that VGF-derived neuropeptides synthesized in the MPNc are effector molecules that increase sexual motivation following induction of sexual arousal in male rats, because VGF knockdown in the MPNc of sexually inexperienced males disrupted an increase in sexual motivation following their first experience of successful copulation (Maejima et al., 2018). Considering these previous studies, the MPNc of male rats may be functional during their first copulation to induce sexual arousal and may be dysfunctional or not necessary for sexual behavior after acquisition of copulatory experience. However, the mechanisms, by which the MPNc of male rats is functional at the first copulation and lower physiological activity at subsequent copulations, has not yet been elucidated.

Higher motivation to copulate, which is reinforced by repeated copulatory experience, is sustained for a long period, because a shorter latency of mount after acquisition of repeated copulatory experience remains unchanged 5 months after the last copulation (Bialy et al., 2000). We hypothesized that plastic changes to synapses in the MPNc occur with copulation, and that these synaptic changes consolidate at structural levels. In this study, to test this hypothesis, we examined the effects of at least two copulatory experiences on the morphology of dendrites and spines in the MPNc and in the MPNlm of male rats. Additionally, to explore the molecular mechanisms of these synaptic changes, we measured the mRNA levels of genes encoding actin-binding proteins in the MPNc and in the MPNlm of sexually naive male rats, males that displayed mount and intromission but not ejaculation, and males that ejaculated for their first and second times.

2. Materials and methods

2.1. Animals

Adult male and female Wistar rats (8–13 weeks old) were used in all experiments. The animals were housed in a room maintained at 22 °C with a 12 h light/12 h dark cycle (lights on: 08:00–20:00) and had free access to a standard diet and tap water. All animal procedures were approved by the Animal Care and Use Committee of Saitama University and were conducted in accordance with the Guidelines for the Care and Use of Experimental Animals of Saitama University.

2.2. Experiment 1: effects of copulatory experience on the morphology of dendrites and spines

2.2.1. Experimental design (Fig. 1A)

A male sexual behavior test was performed three times, resulting in males being categorized into two groups: males ejaculating successfully and having copulatory experience two or three times (hereafter “ejaculators”) and males displaying mount and intromission but not ejaculation (hereafter “non-ejaculators”). The day after the last behavior test, the ejaculators and non-ejaculators were euthanized and the brains collected. In addition, brains were sampled from sexually naive male rats that did not experience contact or copulation with female rats. The brains were subjected to Golgi-Cox staining to visualize dendrites and spines by light microscopy. The morphology of Golgi-Cox stained neurons in the MPNc and in the MPNlm was analyzed to determine the effects of copulatory stimuli on the morphology of dendrites and spines. Blood was sampled from the sexually naive males, non-ejaculators, and ejaculators at the same time as brain harvesting to measure serum testosterone levels.

2.2.2. Male sexual behavior test

Sexual behavior of male rats was observed in a dark room under dimmed red light during the light phase of the light/dark cycle. After acclimatization to the room for 10–15 min, a female in estrus was placed in the home cage (D × W × H: 440 mm × 280 mm × 205 mm) of each male rat. The female rats used for the behavior tests were ovariectomized and subcutaneously injected with estradiol benzoate (20 μg in 0.2 ml sesame oil) and progesterone (500 μg in 0.2 ml sesame oil) 48 h and 4 h before use, respectively. Male rats were then observed for 30 min to record the latency of their first mount, their first intromission, and their first ejaculation, and the number of mounts, intromissions, and ejaculations during each test, and to calculate the incidence of mounts, intromissions, ejaculations in non-ejaculators and ejaculators. The test was conducted three times for each male rat at one-week intervals. The estrous females used in each behavior test were randomly selected so that males did not encounter only a specific female during behavior tests.

2.2.3. Golgi-Cox staining

Golgi-Cox staining was performed using an FD Rapid GolgiStain kit (FD NeuroTechnologies, Columbia, MD, USA). Golgi-Cox-stained 60 μm-thick brain sections were counterstained with 0.1% Cresyl Fast Violet.

2.2.4. Analysis of dendrites and spines

We defined the MPNc and MPNlm of the brain sections with reference to Nissl staining. All neurons whose cell bodies and spiny dendrites were visualized by Golgi-Cox staining and observed in the MPN were selected as the cells of interest. The number of Golgi-Cox-stained neurons were 3–8 cells per animal in the MPNc (naive, 5.2 ± 0.8; non-ejaculator, 4.2 ± 0.5; ejaculator, 4.5 ± 0.7) and 3–6 cells per animal in the MPNlm (naive, 5.2 ± 0.5; non-ejaculator, 4.6 ± 0.5; ejaculator, 4.4 ± 0.4). Golgi-Cox-stained neurons were 3D-reconstructed *in silico* using a light microscope (DM5000B; Leica

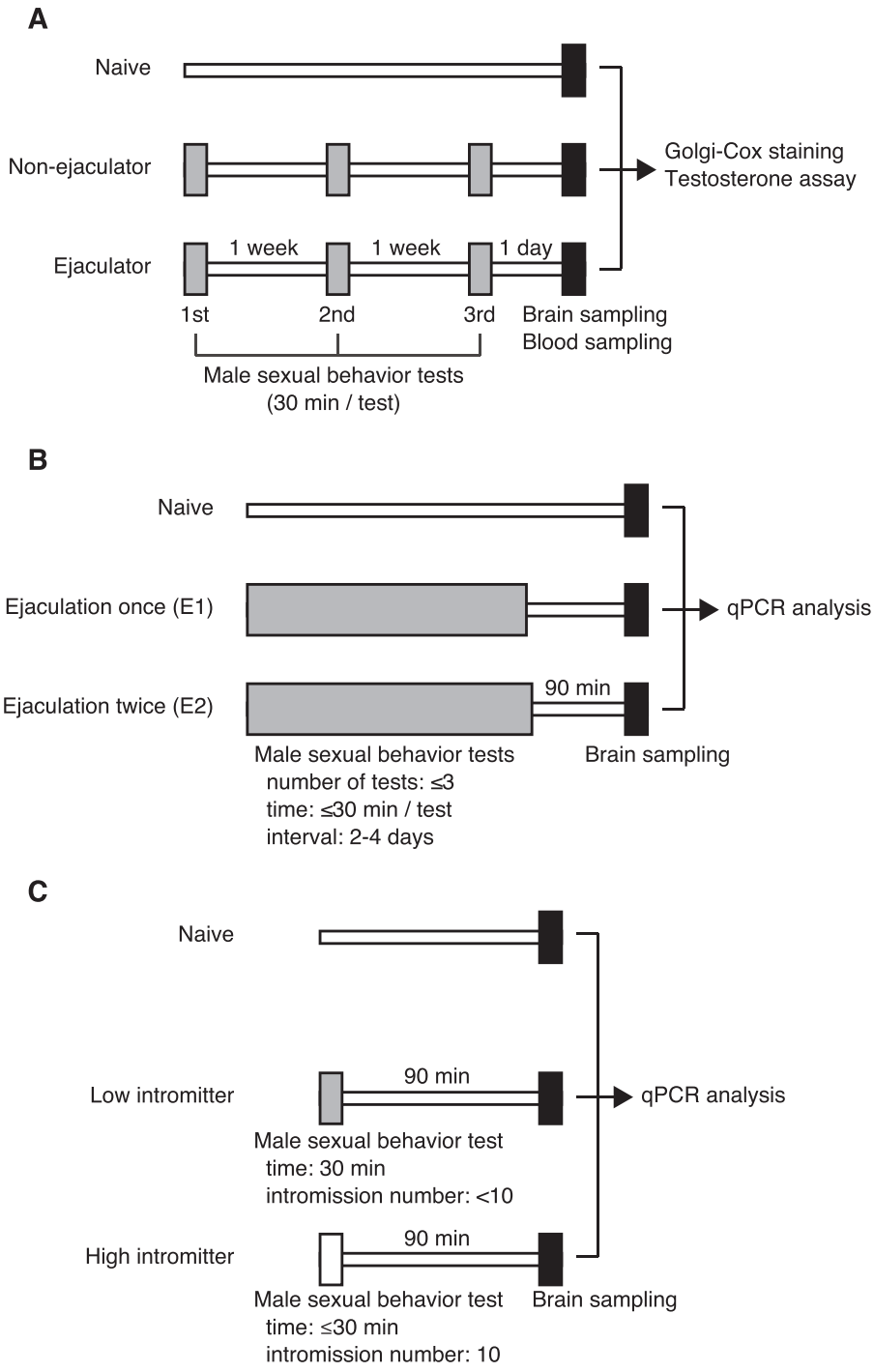

Fig. 1. Schematic illustrations for experimental design. (A) Design of experiment 1 to analyze the effects of copulation on the morphology of dendrites and spines, and serum testosterone levels. There were three experimental groups: sexually naive male rats (Naive) that did not experience contact nor copulation with female rats; non-ejaculators that displayed mounts and intromissions but not ejaculation during 3 successive behavior tests; and ejaculators that encountered a total of 2 or 3 ejaculations within 3 successive behavior tests. (B) Design of experiment 2 to analyze the effects of stimuli from ejaculations on spinogenesis-related gene expression. There were three experimental groups: sexually naive male rats (Naive), males ejaculated once (E1), and males ejaculated twice (E2) during sexual behavior tests. (C) Design of experiment 3 to analyze the effects of stimuli from mounts and intromissions on spinogenesis-related gene expression. There were three experimental groups: sexually naive male rats (Naive), low intromitters that displayed intromissions less than 10 times during a behavior test, and high intromitters that displayed intromissions 10 times during a behavior test.

Microsystems, Wetzlar, Germany) equipped with a charge-coupled device camera (CX9000; MBF Bioscience, Williston, VT, USA) and a computer running Neurolucida software (MBF Bioscience). The outlines of the cell bodies and dendrites were traced, and the number of dendrites and dendritic branches, total length of dendrites, the surface area of dendrites were recorded in each cell. We also recorded the number and locations of the dendritic spines. Spines were categorized into four types (mushroom, filopodia, thin, and stubby) based on their appearance (Bourne and Harris, 2008; Peters and Kaiserman-Abramof, 1970). Specifically, spines having a thick stalk that extends into a large end-bulb, the diameter of which was much greater than the diameter of the stalk, was defined as mushroom spines; spines having a long and thin protrusion from dendrite was defined as filopodia spines; spines having a slender stalk, the length of which was greater than the diameter of the stalk and the diameter of an oval or rounded end-bulb derived from the stalk, was defined as thin spines; and spines that is short and thick protrusion was defined as stubby spines. From the recorded data, we measured the surface area of dendrites per unit length (the recorded values/total length of dendrites), as well as the density of spines (the recorded number of spines/total length of dendrites) for each neuron. The values obtained from the neurons of each animal were averaged to represent the data for each animal.

2.2.5. Testosterone assay

Serum was obtained from blood samples and the lipophilic fraction extracted according to a procedure described previously (Tsukahara

et al., 2009). Testosterone concentrations in the extracted samples were assayed using a Testosterone EIA Kit (Cayman Chemical, Ann Arbor, MI, USA).

2.3. Experiment 2: effects of copulatory stimuli on spinogenesis-related gene expression

2.3.1. Experimental design (Fig. 1B)

A DNA microarray analysis in our previous study showed that gene expression in the MPNc of male rats changed 90 min after they ejaculated for the first and second times and that ejaculation-induced expression of some genes was affected by copulatory experience (Maejima et al., 2018). In this experiment, to verify the hypothesis that stimuli form ejaculation alters the expression of spinogenesis-related genes, a male sexual behavior test was conducted a maximum of three times, and we then collected the brains from male rats that had ejaculated once (E1 males) and twice (E2 males) 90 min after they ejaculated for their first and second times, respectively. We also obtained brains from sexually naive males. Tissue fragments containing the MPNc or the MPNlm were dissected from brain sections. Total RNA was extracted from the tissue fragments, and cDNA was synthesized. The cDNA samples were used for quantitative polymerase chain reaction (qPCR) analysis to measure the mRNA levels of cortactin, drebrin A, cofilin, neurabin, and spinophilin.

2.3.2. Male sexual behavior test

Sexual behavior tests for male rats were conducted by placing an estrous female rat in their home cage under the same conditions as those described in Section 2.2.2. The test was performed a maximum of three times for each male until ejaculation was achieved once or twice. In each test, male rats were monitored for a maximum of 30 min until they ejaculated once. If a male did not ejaculate within 30 min, the test was terminated and they were subjected to another test 2–4 days later.

2.3.3. Sampling of brain tissue fragments

After brains were sampled, they were quickly frozen and then coronally sectioned on a cryostat at a thickness of 40 µm and sections mounted on PEN-membrane slides (Leica Microsystems). The sections were fixed and stained by Nissl staining, and then square-shaped tissue fragments (630 × 630 µm) containing the MPNc or the MPNlm were dissected using a laser microdissection system (LMD 7000; Leica Microsystems) as reported previously (Maejima et al., 2018).

2.3.4. qPCR analysis

Total RNA was extracted from tissue fragments containing the MPNc or the MPNlm and purified using an RNeasy Micro Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized using a Prime Script RT reagent kit (Takara Bio, Shiga, Japan). Standardized samples for quantification were prepared by mixing unknown cDNA samples and serially diluting them in EASY Dilution (Takara Bio). qPCR was performed using a Light Cycler ST300 (Roche Diagnostics, Mannheim, Germany). Two microliters of standards or unknown samples were amplified in a 20 µL reaction mixture containing 200 nM of each gene-specific primer (see Table 1) and 10 µL 2 × SYBR Premix Ex Taq (Takara Bio). The mRNA levels of the target genes were normalized against the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

2.4. Experiment 3: effects of stimuli from mounts and intromissions on spinogenesis-related gene expression

2.4.1. Experimental design (Fig. 1C)

In this experiment, we investigated whether copulatory stimuli, excluding stimuli from ejaculation, affect the expression of spinogenesis-related genes or not. We performed a male sexual behavior test, resulting in male rats being categorized into two groups: males that displayed intromissions 10 times within 30 min (hereafter “high intromitters”) and males that displayed intromissions less than 10 times for 30 min (hereafter “low intromitters”). Brains were sampled from the high intromitters 90 min after they intromitted for the tenth time and from the low intromitters 90 min after the end of the behavioral test. We also obtained brains from sexually naive males that were placed alone in their home cage for 120 min under the same conditions as observation of sexual behavior. The brains of sexually naive, high intromitters, and low intromitters harvested for qPCR analysis. Tissue fragments containing the MPNc or the MPNlm were dissected from brain sections, and total RNA was extracted from the tissue fragments. The cDNA samples were used for qPCR analysis to measure the mRNA levels of cortactin, drebrin A, cofilin, neurabin, and spinophilin.

2.4.2. Male sexual behavior test

A sexual behavior test for male rats was conducted by placing an estrous female rat in their home cage under the same conditions as those described in Section 2.2.2. The test was performed for a maximum of 30 min until male rats intromitted 10 times to obtain the high intromitters or for 30 min to obtain the low intromitters displaying intromissions less than 10 times.

2.4.3. Sampling of brain tissue fragments

Tissue fragments of the MPNc and MPNlm were sampled from Nissl-stained brain sections using the same procedure as described in Section 2.3.3.

2.4.4. qPCR analysis

Total RNA extraction, cDNA synthesis, and standardized sample preparation were carried out using the same procedure as described in Section 2.3.4. qPCR was performed using a LightCycler 96 System (Roche Diagnostics). One microliters of standards or unknown samples were amplified in a 10 µL reaction mixture containing 400 nM of each gene-specific primer (see Table 1) and 5 µL 2 × TB Green Premix Ex Taq II (Tli RNaseH Plus) (Takara Bio). The mRNA levels of the target genes were normalized against the level of GAPDH mRNA.

2.5. Statistical analysis

Two-way analysis of variance (ANOVA) was used to assess the differences in the number of mounts, intromissions, and ejaculations between non-ejaculators and ejaculators and the effects of the number of sexual behavior tests. Additionally, 2-way ANOVA was used to assess the differences in dendrite morphology and spine density between the

Table 1
Sequences of primers used for quantitative polymerase chain reaction analysis.

Target gene	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
cortactin	GGGCTTTGACTACCAGGG	AGTCTTTCTGGGACTCATGC
drebrin A	GGCTGTGCCAACCTTCTT	GCTCCAACACCTGATCTACC
cofilin	AGACCAAGTCCCGTCCT	AGGGTCATTTCCACAGTTCA
neurabin	AAGCAGCAGAGAATGAGAAAGT	GGTCTCTTTGAGATGCTCGT
spinophilin	ACGAGCTGGAGAAGCGA	CACCCTCAGTCACAGTCTTG
GAPDH	GTGGAGTCTACTGGCGT	ACAATCTTGAGGGAGTTGTC

MPNc and MPNlm and among sexually naive males, non-ejaculators, and ejaculators. One-way ANOVA was performed to determine differences in dendrite morphology, spine density, and serum testosterone concentration among sexually naive males, non-ejaculators, and ejaculators. One-way ANOVA was also performed to determine differences in the mRNA levels of the genes for actin-binding proteins among sexually naive males, E1 males, and E2 males, and among sexually naive males, low intromitters, and high intromitters. When significant overall effects were detected by one-way ANOVA, Fisher's least significant difference test was performed as a post hoc test. Student's *t*-test was used to assess differences in the latency of mount and intromission between non-ejaculators and ejaculators and in the latency of mount and intromission between high intromitters and low intromitters. Chi-square test was carried out to compare the incidence of mounts and intromissions in non-ejaculators with that in ejaculators.

3. Results

3.1. Experiment 1: effects of copulatory experience on the morphology of dendrites and spines

3.1.1. Male sexual behavior in non-ejaculators and ejaculators

We separated test animals into two groups of non-ejaculators and ejaculators according to performance in the sexual behavior tests. Overall, the ejaculators displayed more mounts and intromissions compared with the non-ejaculators. Consequently, the ejaculators encountered a total of 2 or 3 ejaculations within the 3 successive behavior tests, while the non-ejaculators did not experienced ejaculation during the tests.

The number of mounts was significantly larger ($F_{(1,30)} = 5.46$, $p = 0.026$) in the ejaculators than in the non-ejaculators and was significantly increased ($F_{(2,30)} = 3.63$, $p = 0.039$) with repeating behavior tests (Fig. 2A). The number of mounts was not significantly affected by the interaction of main factors ($F_{(2,30)} = 1.01$, $p = 0.38$). There was no significant difference in the incidence of mount (1st test: $\chi^2_{(1)} = 0.00$, $p = 1.00$; 2nd test: $\chi^2_{(1)} = 2.40$, $p = 0.12$; 3rd test: $\chi^2_{(1)} = 2.40$, $p = 0.12$) and the latency of mount (1st test: $t_{(8)} = 0.27$, $p = 0.79$; 2nd test: $t_{(8)} = 1.15$, $p = 0.28$; 3rd test: $t_{(8)} = 0.97$, $p = 0.36$) between the non-ejaculators and ejaculators (Table 2).

The number of intromissions was significantly larger ($F_{(1,30)} = 19.41$, $p = 0.00012$) in ejaculators compared with non-ejaculators (Fig. 2B). The number of intromissions did not significantly ($F_{(2,30)} = 1.64$, $p = 0.21$) increase with the number of behavior test performances, although the number of intromission in non-ejaculators, but not in ejaculators, increased slightly. The number of intromissions was not significantly affected by the interaction of main factors ($F_{(2,30)} = 0.43$, $p = 0.65$). In the 1st and 3rd male sexual behavior tests, there was no significant difference in the incidence of intromission (1st test: $\chi^2_{(1)} = 1.33$, $p = 0.25$; 3rd test: $\chi^2_{(1)} = 2.40$, $p = 0.12$) and the latency of intromission (1st test: $t_{(4)} = 0.47$, $p = 0.67$; 3rd test: $t_{(8)} = 1.32$, $p = 0.22$) between non-ejaculators and ejaculators (Table 2). However, in the 2nd male sexual behavior test, ejaculators showed significantly higher incidence of intromissions ($\chi^2_{(1)} = 4.00$, $p = 0.046$) and significantly shorter latency of intromission ($t_{(7)} = 2.41$, $p = 0.047$) compared with non-ejaculators.

Ejaculators had a significantly greater ($F_{(1,30)} = 54.44$, $p = 0.000000032$) number of ejaculations during behavior tests than did non-ejaculators (Fig. 2C). There was no significant effect of the number of behavior test performances ($F_{(2,30)} = 0.28$, $p = 0.76$) and the interaction of main factors ($F_{(2,30)} = 0.28$, $p = 0.76$) on the number of ejaculations. The incidence of ejaculations was significantly higher in the ejaculators than in non-ejaculators (1st test: $\chi^2_{(1)} = 6.00$, $p = 0.014$; 2nd test: $\chi^2_{(1)} = 8.57$, $p = 0.0034$; 3rd test: $\chi^2_{(1)} = 8.57$, $p = 0.0034$; Table 2).

3.1.2. Morphology of dendrites and spine density in the MPNc

Most Golgi-Cox-stained MPNc neurons had 2–3 dendrites that branched once or not (Fig. 3A). Dendrite morphology in Golgi-Cox-stained MPNc neurons did not differ among sexually naive males and non-ejaculators and ejaculators (Fig. 3B–D). There was no significant

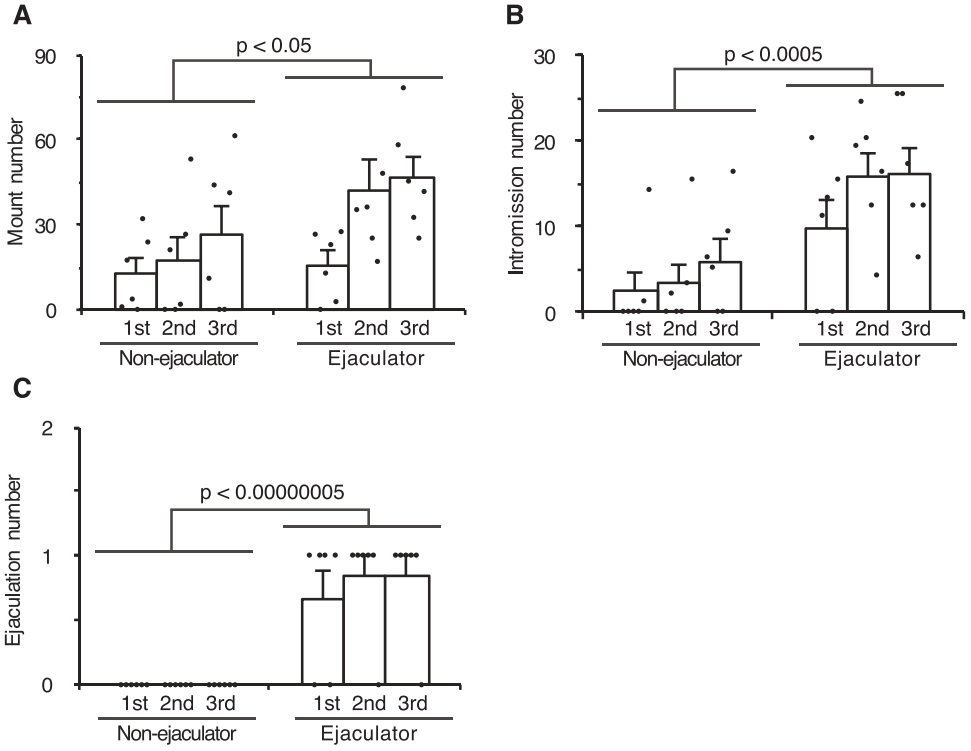

Fig. 2. Differences in the male sexual performance test between non-ejaculators and ejaculators. The number of mounts (A), number of intromissions (B), and number of ejaculations (C) in non-ejaculators and ejaculators. Data are the mean ± SEM; black dots represent individual data; n = 6 for each group.

Table 2
The performance of male sexual behavior tests in non-ejaculators and ejaculators.

	1 st test		2nd test		3rd test	
	Non-ejaculator	Ejaculator	Non-ejaculator	Ejaculator	Non-ejaculator	Ejaculator
Incidence						
Mount	5/6	5/6	4/6	6/6	4/6	6/6
Intromission	2/6	4/6	3/6	6/6*	4/6	6/6
Ejaculation	0/6	4/6*	0/6	5/6**	0/6	5/6**
Latency						
Mount	186.6 ± 45.7	166.8 ± 56.2	381.3 ± 251.5	53.0 ± 20.8	238.8 ± 209.7	12.3 ± 3.0
Intromission	292.5 ± 212.5	207.3 ± 85.8	813.0 ± 339.9	169.7 ± 100.2*	347.5 ± 180.7	69.5 ± 30.8
Ejaculation	–	971.5 ± 181.7	–	1009.2 ± 201.0	–	1035.6 ± 155.9

Values of latency are the mean ± SEM. *, $p < 0.05$ vs. non-ejaculator; **, $p < 0.01$ vs. non-ejaculator.

difference in the number of dendrites ($F_{(2,15)} = 0.60$, $p = 0.56$) and dendritic branches ($F_{(2,15)} = 0.56$, $p = 0.58$), and the surface area of dendrites ($F_{(2,15)} = 0.45$, $p = 0.64$) in Golgi-Cox-stained MPNc neurons.

There were fewer dendritic spines on Golgi-Cox-stained MPNc neurons in ejaculators compared with those in sexually naive males and non-ejaculators (Fig. 4A). The density of mushroom spines in the MPNc significantly differed among sexually naive males, non-ejaculators and ejaculators ($F_{(2,15)} = 8.58$, $p = 0.0033$). The density of mushroom spines in ejaculators was significantly lower than in sexually naive males ($p < 0.05$) and non-ejaculators ($p < 0.01$) (Fig. 4B). However, the densities of filopodia, thin, or stubby spines did not differ significantly among sexually naive males and non-ejaculators and ejaculators (filopodia: $F_{(2,15)} = 0.77$, $p = 0.48$; thin: $F_{(2,15)} = 0.055$, $p = 0.95$; stubby: $F_{(2,15)} = 0.79$, $p = 0.47$; Fig. 4C–E).

3.1.3. Morphology of dendrites and spine density in the MPNlm

Like Golgi-Cox-stained neurons in the MPNc, most Golgi-Cox-stained neurons in the MPNlm had 2–3 dendrites that branched once or not (Fig. 5A). There was no significant difference in the number of dendrites ($F_{(2,13)} = 0.19$, $p = 0.83$) and the number of dendritic branches ($F_{(2,13)} = 0.62$, $p = 0.56$) among sexually naive male rats,

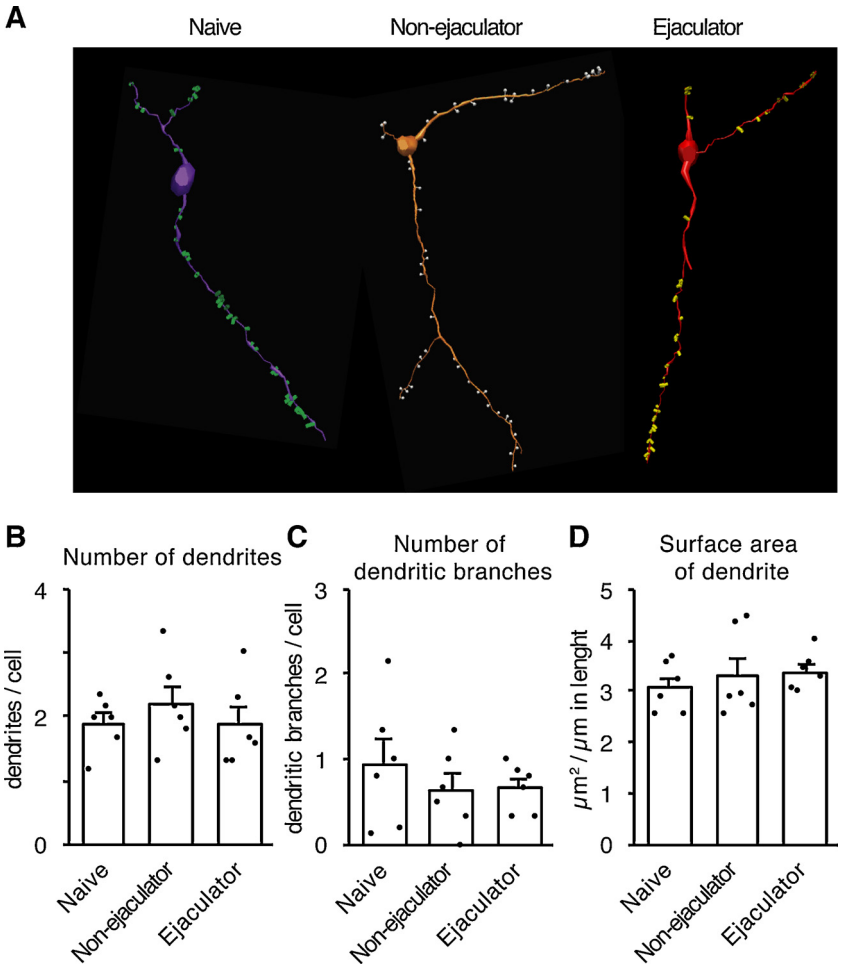

Fig. 3. The morphology of spiny dendrites in the MPNc did not change with copulation. (A) Three-dimensional reconstruction of representative Golgi-Cox-stained neurons in the MPNc of a sexually naive male (Naive), a non-ejaculator, and an ejaculator. The number of dendrites (B), number of dendritic branches (C), and surface area of dendrites (D) in Naive males, non-ejaculators, and ejaculators. Data are the mean ± SEM; black dots represent individual data; n = 6 for each group.

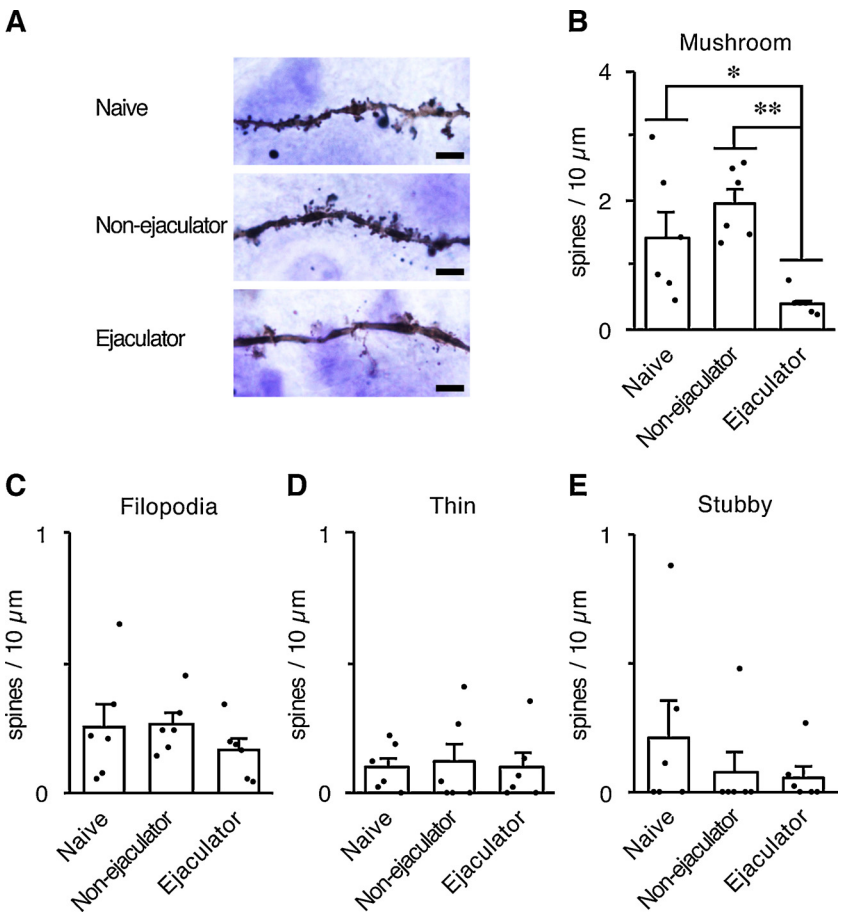

Fig. 4. Dendritic spine changes in the MPNc caused by repeated experience of successful copulation. (A) Representative photomicrographs of dendritic spines in the MPNc of a sexually naive male rat (Naive), a non-ejaculator, and an ejaculator. Scale bars, 5 μm. Density of mushroom spines (B), filopodia spines (C), thin spines (D), and stubby spines (E) in the MPNc of Naive, non-ejaculators, and ejaculators. Data are the mean ± SEM; black dots represent individual data; n = 6 for each group. *, $p < 0.05$; **, $p < 0.01$.

non-ejaculators, and ejaculators (Fig. 5B and C). However, there were significant differences in the surface area of dendrites ($F_{(2,13)} = 4.09$, $p = 0.042$). Ejaculators had significantly ($p < 0.05$) larger surface area of dendrites in the MPNlm compared with sexually naive males and non-ejaculators (Fig. 5D).

In the MPNlm there was no striking difference in the density of spines among sexually naive male rats, non-ejaculators, and ejaculators (Fig. 6). We did not find any significant difference in the density of mushroom spines ($F_{(2,13)} = 0.32$, $p = 0.73$), filopodia spines ($F_{(2,13)} = 0.22$, $p = 0.81$), thin spines ($F_{(2,13)} = 0.80$, $p = 0.47$), and stubby spines ($F_{(2,13)} = 1.13$, $p = 0.35$).

When compared the morphology of dendrites and the spine density in the MPNlm with those in the MPNc, Golgi-Cox-stained MPNlm neurons had significantly larger surface area of dendrites ($F_{(1,28)} = 4.61$, $p = 0.041$), significantly lower density of filopodia spines ($F_{(1,28)} = 7.19$, $p = 0.012$), and significantly higher density of stubby spines ($F_{(1,28)} = 7.02$, $p = 0.013$). There was no significant difference in the number of dendrites ($F_{(1,28)} = 0.42$, $p = 0.52$) and dendritic branches ($F_{(1,28)} = 0.083$, $p = 0.78$), and the density of mushroom spines ($F_{(1,28)} = 3.72$, $p = 0.064$) or thin spines ($F_{(1,28)} = 0.49$, $p = 0.49$) between the MPNlm and MPNc.

3.1.4. Testosterone level

The concentrations of serum testosterone were 4.99 ± 1.10 ng/mL in sexually naive male rats, 3.31 ± 0.72 ng/mL in non-ejaculators, and 2.16 ± 0.65 ng/mL in ejaculators. There was no significant difference in serum testosterone concentration among the groups ($F_{(2,15)} = 2.81$, $p = 0.092$).

3.2. Experiment 2: effects of copulatory stimuli on spinogenesis-related gene expression

The animals used in this experiment were sexually naive male rats that had not had any contact with nor copulated with female rats, and sexually experienced male rats that had ejaculated once (E1 males) or twice (E2 males) [see (Maejima et al., 2018) for details of behavior tests performance].

In the MPNc, the mRNA level of drebrin A significantly differed among sexually naive males, E1 and E2 males ($F_{(2,12)} = 8.68$, $p = 0.0047$). The mRNA level of drebrin A in the MPNc was significantly lower ($p < 0.01$) in E1 and E2 males than in sexually naive males (Fig. 7A). The mRNA level of spinophilin in the MPNc significantly differed among the groups ($F_{(2,12)} = 7.22$, $p = 0.0087$). The spinophilin mRNA level in the MPNc was significantly lower in E1 males ($p < 0.05$) and E2 males ($p < 0.01$) than in sexually naive males (Fig. 7B). There was significant difference in the mRNA level of cofilin in the MPNc among the groups ($F_{(2,12)} = 4.22$, $p = 0.041$), and it was significantly higher ($p < 0.05$) in E2 males than in E1 males (Fig. 7C). In the MPNc, there was no significant difference in the mRNA levels of cortactin ($F_{(2,12)} = 3.69$, $p = 0.056$) and neurabin ($F_{(2,12)} = 0.62$, $p = 0.56$) among sexually naive, E1 males, and E2 males (Fig. 7D and E).

In the MPNlm, there was no significant difference in the mRNA levels of drebrin A ($F_{(2,12)} = 0.58$, $p = 0.57$), spinophilin ($F_{(2,12)} = 0.98$, $p = 0.40$), cofilin ($F_{(2,12)} = 1.58$, $p = 0.25$), and neurabin ($F_{(2,12)} = 3.74$, $p = 0.055$) among sexually naive, E1 males, and E2 males (Fig. 8A, B, C, E). The mRNA levels of cortactin in the MPNlm significantly differed among the groups ($F_{(2,12)} = 4.69$, $p = 0.031$). The cortactin mRNA level in E1 males was significantly higher ($p < 0.05$) than in sexually naive and E2 males (Fig. 8D).

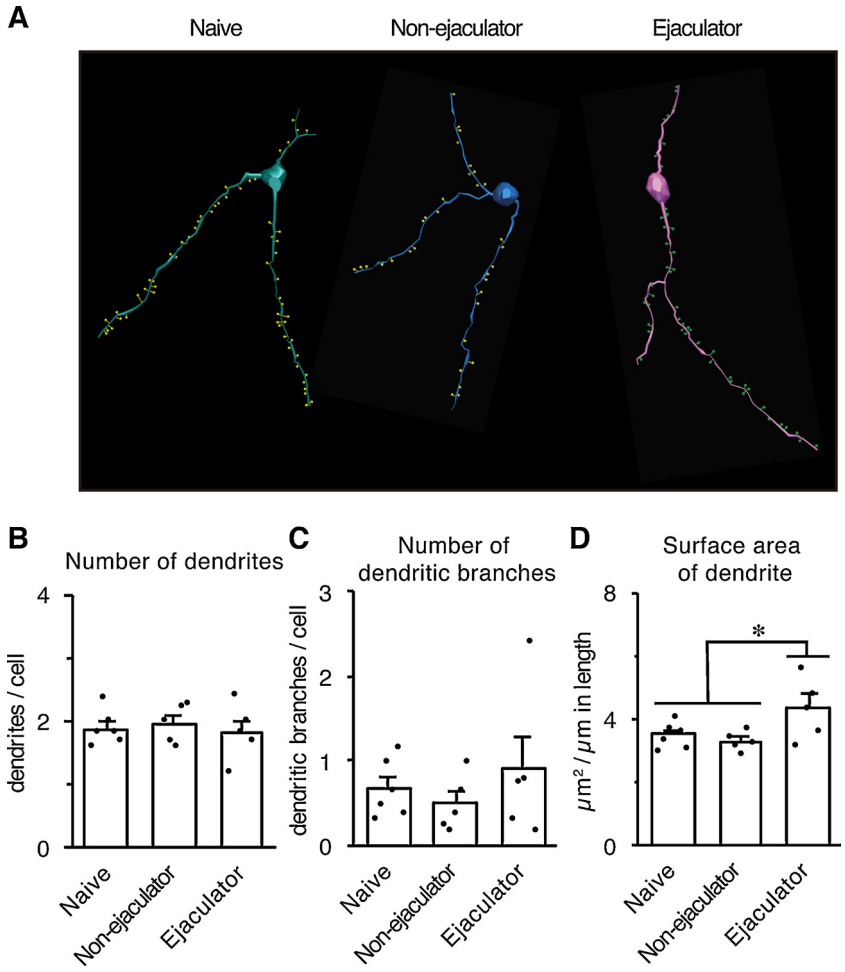

Fig. 5. Morphological changes in spiny dendrites in the MPNlm caused by repeated experience of successful copulation. (A) Three-dimensional reconstruction of representative Golgi-Cox-stained neurons in the MPNlm of a sexually naive male (Naive), a non-ejaculator, and an ejaculator. The number of dendrites (B), number of dendritic branches (C), and surface area of dendrites (D) in Naive males, non-ejaculators, and ejaculators. Data are the mean ± SEM; black dots represent individual data; n = 6 for Naive; n = 5 for non-ejaculators; n = 5 for ejaculators. *, $p < 0.05$.

3.3. Experiment 3: effects of stimuli from mounts and intromissions on spinogenesis-related gene expression

3.3.1. Male sexual behavior in low intromitters and high intromitters

In the result of a male sexual behavior test, which was performed for a maximum of 30 min, the latency of mount and intromission in high intromitters were 92.2 ± 36.2 s and 157.3 ± 41.3 s, respectively, and the latency of mount and intromission in low intromitters were 403.2 ± 237.4 s and 553.8 ± 253.5 s, respectively. Although the high intromitters more quickly displayed mount and intromission compared with the low intromitters, there was no significant difference in the latency of mount ($t_{(10)} = 1.30$, $p = 0.22$) and intromission ($t_{(10)} = 1.54$, $p = 0.15$) between high and low intromitters. The number of mounts and intromissions in high intromitters were 2.5 ± 0.72 and 10, respectively, and the number of mounts and intromissions in low intromitters were 3.33 ± 0.84 and 2.67 ± 0.95, respectively.

3.3.2. Spinogenesis-related gene expression

In the MPNc, there was no significant difference in the mRNA levels of drebrin A ($F_{(2,15)} = 3.31$, $p = 0.065$), spinophilin ($F_{(2,15)} = 1.25$, $p = 0.32$), cofilin ($F_{(2,15)} = 0.33$, $p = 0.72$), cortactin ($F_{(2,15)} = 0.45$, $p = 0.65$), and neurabin ($F_{(2,15)} = 1.86$, $p = 0.19$) among sexually naive males, low intromitters, and high intromitters (Fig. 9). Like the MPNc, the MPNlm did not exhibit significant difference in the mRNA levels of drebrin A ($F_{(2,15)} = 0.80$, $p = 0.47$), spinophilin ($F_{(2,15)} = 0.77$, $p = 0.48$), cofilin ($F_{(2,15)} = 1.03$, $p = 0.38$), cortactin ($F_{(2,15)} = 0.81$, $p = 0.46$), and neurabin ($F_{(2,15)} = 1.01$, $p = 0.39$) among the experimental groups (Fig. 10).

4. Discussion

The current study demonstrated that the density of mushroom spines in the MPNc of ejaculators that had experienced successful copulation 2–3 times was significantly less than that of sexually naive male rats and non-ejaculators that did not achieve ejaculation. These findings may indicate that mushroom spines in the MPNc of male rats are pruned after they have experienced successful copulation with ejaculation at least twice. Pruning mushroom spines in dendrites appears to be confined to the MPNc, because we observed no significant differences in spine density in the MPNlm among sexually naive males and non-ejaculators and ejaculators. Our previous analysis of c-Fos indicated that the MPNc of male rats contains a neuronal population that activates during the first copulation and then remains silent during later copulations (Yamaguchi et al., 2018). Considering that mushroom spines are the postsynaptic component for excitatory neurotransmission (Bourne and Harris, 2008; Tonnesen and Nagerl, 2016), the reduced mushroom spine density after copulatory experience may be responsible for the reduction of neuronal activity in the MPNc of sexually experienced males. However, it is also possible that factors other than glutamatergic signaling mediate the reduction of mushroom spine density and neuronal activity in the MPNc after copulatory experience.

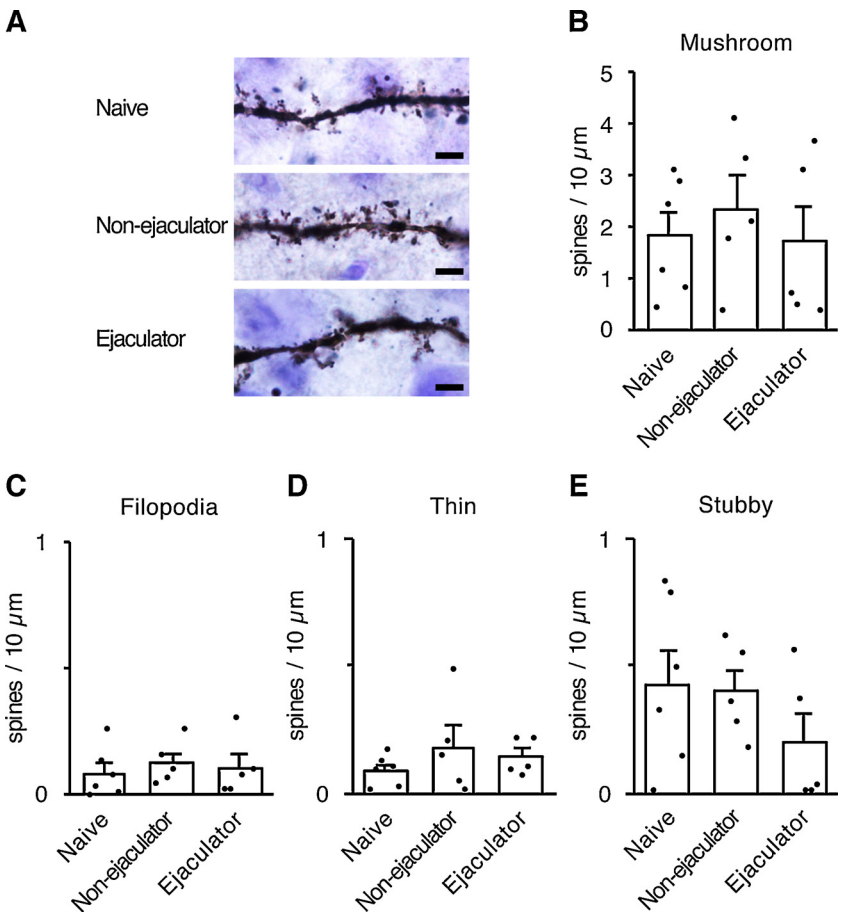

Fig. 6. The density of dendritic spines in the MPNlm did not change with copulation. (A) Representative photomicrographs of dendritic spines in the MPNlm of a sexually naive male rat (Naive), a non-ejaculator, and an ejaculator. Scale bars, 5 μm. Density of mushroom spines (B), filopodia spines (C), thin spines (D), and stubby spines (E) in the MPNlm of Naive, non-ejaculators, and ejaculators. Data are the mean ± SEM; black dots represent individual data; n = 6 for Naive; n = 5 for non-ejaculators; n = 5 for ejaculators.

Further investigations are required to determine the direct factors to reduce spine density and neuronal activity in the MPNc after copulatory experience.

The physiological significance of reduced neuronal activity following pruning of mushroom spines in the MPNc has not yet been clarified. However, lesions of the MPNc adversely affect sexual behavior in sexually naive males (De Jonge et al., 1989) but not in sexually experienced males that have ejaculated at least twice (Arendash and Gorski, 1983). Additionally, VGF-derived neuropeptides synthesized in the MPNc serve as effector molecules to increase sexual motivation

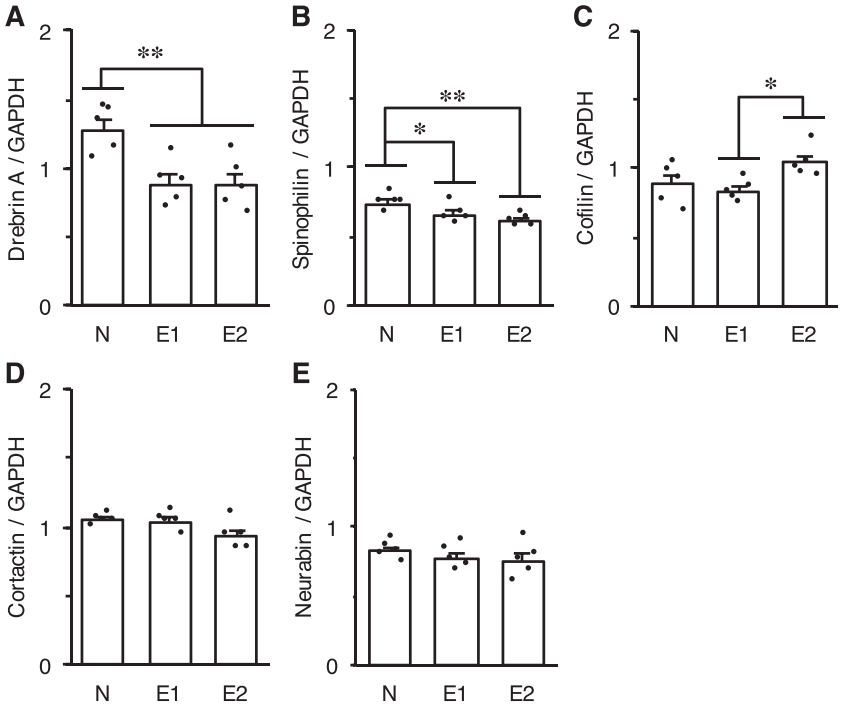

Fig. 7. Effects of copulatory stimuli with ejaculation on the expression of genes encoding actin-binding proteins related to spinogenesis in the MPNc. The mRNA levels of drebrin A (A), spinophilin (B), cofilin (C), cortactin (D), and neurabin (E) in the MPNc in sexually naive male rats (N) and male rats that ejaculated once (E1) and twice (E2). Data are the mean ± SEM; black dots represent individual data; n = 5 for each group. *, $p < 0.05$; **, $p < 0.01$.

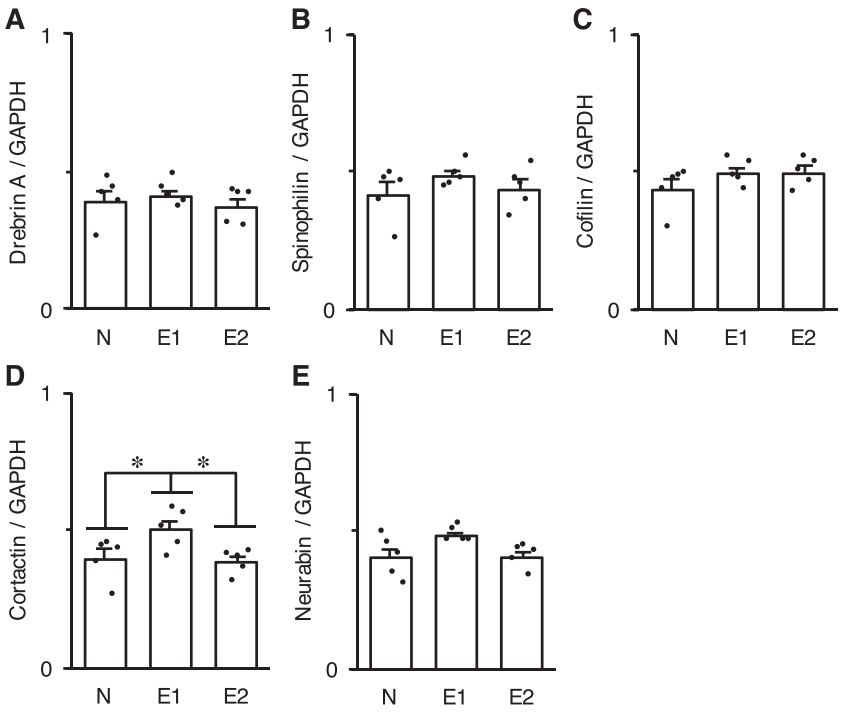

Fig. 8. Effects of copulatory stimuli with ejaculation on the expression of genes encoding actin-binding proteins related to spinogenesis in the MPNlm. The mRNA levels of drebrin A (A), spinophilin (B), cofilin (C), cortactin (D), and neurabin (E) in the MPNlm in sexually naive male rats (N) and male rats that ejaculated once (E1) and twice (E2). Data are the mean ± SEM; black dots represent individual data; n = 5 for each group. *, $p < 0.05$.

after copulatory experience in male rats, because knockdown of VGF in the MPNc of sexually inexperienced males disrupts an increased preference for estrous females and a reduction of the latency of mount and of intromission following copulatory experience (Maejima et al., 2018). These findings may indicate that the MPNc of male rats is functional during their first copulation to induce sexual arousal, which is followed by more efficient sexual behavior, and is not necessary or less functional for male sexual behavior after acquisition of copulatory experience. A transient activation of MPNc neurons during the first copulation and subsequent silencing the MPNc neuron activity during subsequent copulation and pruning of mushroom spines, may be a mechanism for the induction of sexual arousal in male rats. However, further investigations are required to clarify the details of the mechanism for sexual arousal induction.

The MPN modulates the changes in sexual motivation and performance that result from the induction of sexual arousal and the acquisition of sexual experience in male rats. According to a model proposed to explain the mechanisms (Hull and Dominguez, 2006; Will et al., 2014), glutamate and dopamine play a critical role in sensitizing the MPN to sexual stimuli. Glutamate is released in the MPN during copulation (Dominguez et al., 2006b) and acts via NMDA receptors to facilitate sexual behavior in male rats (Dominguez et al., 2007; Vigdorchik et al., 2012). Dopamine is also released in the MPN during copulation (Hull et al., 1995) and affect the MPN to facilitate sexual

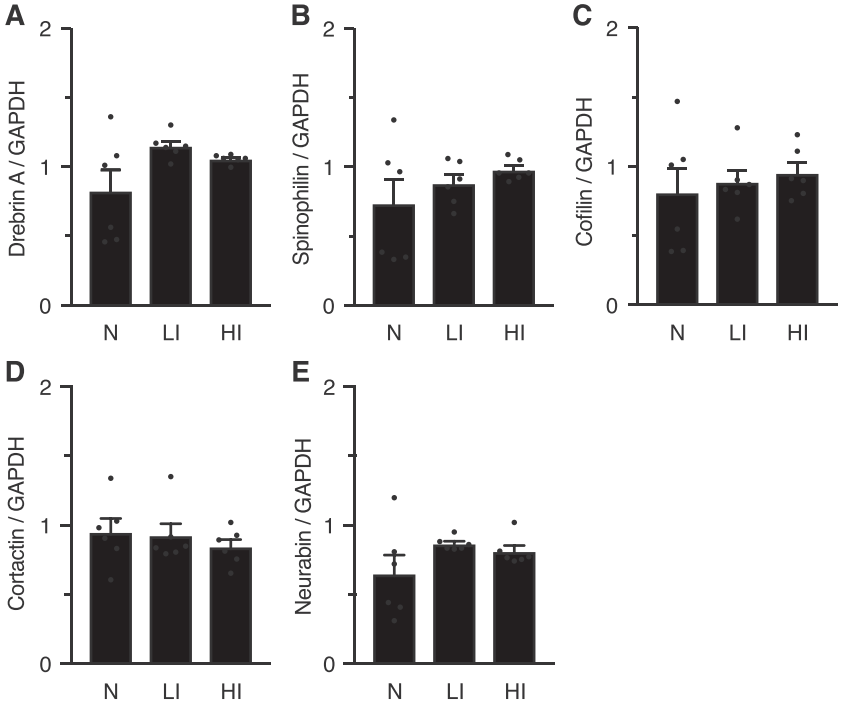

Fig. 9. Stimuli from mounts and intromissions did not change the expression of genes encoding actin-binding proteins related to spinogenesis in the MPNc. The mRNA levels of drebrin A (A), spinophilin (B), cofilin (C), cortactin (D), and neurabin (E) in the MPNc in sexually naive male rats (N), low intromitters (LI), and high intromitters (HI). Data are the mean ± SEM; black dots represent individual data; n = 6 for each group.

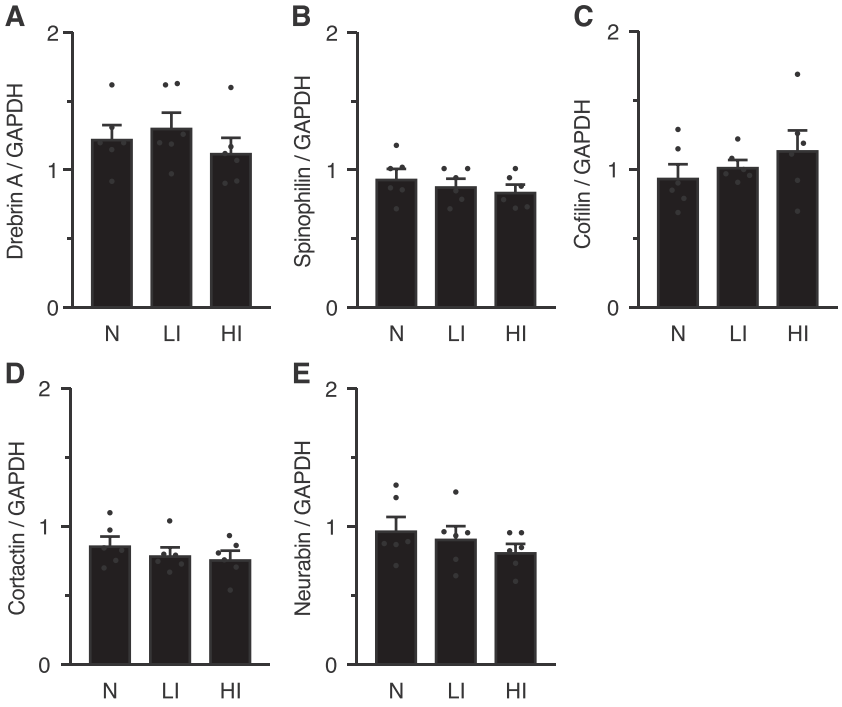

Fig. 10. Stimuli from mounts and intromissions did not change the expression of genes encoding actin-binding proteins related to spinogenesis in the MPNlm. The mRNA levels of drebrin A (A), spinophilin (B), cofilin (C), cortactin (D), and neurabin (E) in the MPNlm in sexually naive male rats (N), low intromitters (LI), and high intromitters (HI). Data are the mean ± SEM; black dots represent individual data; n = 6 for each group.

behavior in male rats (Dominguez and Hull, 2005). Additionally, nitric oxide (NO), which also acts in the MPN to facilitate male sexual behavior (Lagoda et al., 2004), serves as a downstream signal for the action of glutamate via NMDA receptors to stimulate dopamine release in the MPN (Dominguez et al., 2004; Sato and Hull, 2006). In this model, sensitization of the MPN to sexual stimuli following acquisition of sexual experience is explained by an increase in the open probability of NMDA receptors by phosphorylation and an increase in NO production by upregulating the expression of NO synthase, as mating increases phosphorylation of NMDA receptors in the MPN of male rats (Dominguez et al., 2007) and NO synthase expression in the MPN of male rats increases in response to sexual experience (Dominguez et al., 2006a; Nutsch et al., 2014). Based upon the previously proposed model, postsynaptic neurons receiving glutamatergic and dopaminergic inputs are likely sensitized to sexual stimuli. However, our present and previous studies (Maejima et al., 2018; Yamaguchi et al., 2018) suggest that the MPNc contains neurons that are activated during the first copulation but not later copulations. A previous study reported that the number of MPN neurons that express dopamine D2 receptors and are activated during copulation is larger in sexually naive males than in sexually experienced males (Nutsch et al., 2016), suggesting that the MPN contains not only neurons that are sensitized to sexual stimuli after sexual experience but also neurons whose activity during copulation decreases after sexual experience. Our present and previous studies (Maejima et al., 2018; Yamaguchi et al., 2018) supports the idea that neurons activated transiently during the first copulation are localized in the MPNc.

Actin is a major cytoskeletal component of dendritic spines (Landis and Reese, 1983). Regulation of the actin cytoskeleton is essential for spine morphology and structural synaptic plasticity (Carlisle and Kennedy, 2005; Matus, 2000; Tada and Sheng, 2006). We therefore measured the mRNA levels of several genes that encode actin-binding proteins in the MPNc of sexually naive males and males that have experienced ejaculation once or twice to determine whether copulatory stimuli with ejaculation affect the gene expression of actin-binding proteins. We found that the mRNA levels of drebrin A and spinophilin in the MPNc were significantly lower in male rats after they ejaculated for their first and second times than in sexually naive males. However, in the MPNlm the mRNA levels of drebrin A and spinophilin in males ejaculated for the first and second times did not significantly differ from those in sexually naive males. These finding indicate that down-regulation of actin-binding protein gene expression after copulatory experience occurs at the MPNc but not at the MPNlm. This is consistent with the morphological analysis of Golgi-Cox-stained neurons showing that pruning of mushroom spines after copulatory experience is a phenomenon specific to the MPNc. It is well known that actin-binding proteins play an important role in the formation and function of dendritic spines. Knockdown of drebrin A attenuates synaptic clustering of postsynaptic density-95, which is involved in synaptic plasticity and dendritic spine stabilization (Sala and Segal, 2014), and clustering of drebrin A and filamentous-actin in cultured hippocampal neurons (Takahashi et al., 2003). Overexpression of drebrin A alters the shape of spines in cultured cortical neurons by increasing their length (Hayashi and Shirao, 1999). Deletion of the spinophilin gene causes an increase in spine density on caudatoputamen neurons and hippocampal neurons during development in mice (Feng et al., 2000). Additionally, spinophilin knockout mice show dysregulated glutamate receptor currents and reduced long-term depression (Feng et al., 2000). Thus, drebrin A and spinophilin play pivotal roles in spine morphogenesis. Down-regulation of the genes expressing these molecules by copulation may be a mechanism underlying the reduced number of spines in the MPNc of male rats after they have experienced copulation.

We unexpectedly found that the mRNA level of cofilin in the MPNc was higher in E2 males than in E1 males, although the levels in both E1 and E2 males did not significantly differ from that in sexually naive males. In addition, the mRNA levels of cortactin in the MPNlm was higher in E1 males than in sexually naive and E2 males. It is largely unclear what these findings mean physiologically. According to the results of the morphological analysis of neurites and spines in this study, it is likely that the changes in the mRNA of cofilin in the MPNc and of cortactin in the MPNlm by copulation do not affect the morphology of MPNc neurons and MPNlm neurons respectively.

As discussed before, we suggested that pruning mushroom spines in the MPNc occurs after male rats have experienced ejaculation, because the density of mushroom spine in the MPNc of ejaculators was significantly lower than those of non-ejaculators. In addition, we showed that copulatory stimuli with ejaculation significantly reduces the mRNA levels of drebrin A and spinophilin in the MPNc of male rats. However,

it is not able to exclude the possibility that the intrinsic performance of sexual behavior before copulatory experience differs between the non-ejaculators and ejaculators, which may reflect the difference in mushroom spine density between the non-ejaculators and ejaculators. According to the results of sexual behavior tests, ejaculators displayed their first intromission more quickly and more intromissions than non-ejaculators. In the first behavior test, 4 of 6 ejaculators ejaculated following intromissions more than 10 times, while 5 of 6 non-ejaculators displayed few intromissions. We therefore carried out a sexual behavior test to obtain male rats that have higher sexual performance and experienced intromissions 10 times (high intromitters corresponding potentially to ejaculators) and males that have lower sexual performance and exhibit only a few intromissions (low intromitters corresponding potentially to non-ejaculators) and then measured the mRNA levels of several genes that encode actin-binding proteins, including drebrin A and spinophilin. As a result, the mRNA levels of the genes in the MPNc of the low and high intromitters did not differ from each other and showed a comparable level to sexually naive males. This finding supports the notion that a decrease in the mRNA levels of drebrin A and spinophilin in the MPNc, which may be followed by decreasing mushroom spine density, occurs when male rats have experienced copulation with ejaculation.

It has been demonstrated that sexual experience influences the medial preoptic area (MPA), including the MPN, in male mice. Sexual experience increases the density of mushroom spines and the expression of PSD-95 in the MPA of male mice (Jean et al., 2017). Additionally, sexual experience upregulates the expression of GluN1, a subunit of the NMDA glutamate receptor, in the male MPA (Jean et al., 2017). These findings suggest that an increase in synaptic number and excitatory neurotransmission via the glutamatergic system in response to sexual experience are related to greater efficiency of male sexual behavior following sensitization of the MPA to sexual stimuli. However, our present study using male rats showed that the density of spines in the MPNlm was not significantly affected by copulatory experience but that the density of mushroom spines in the MPNc significantly decreased after copulatory experience. According to anatomical studies in rats and mice, the MPN of rats is composed of three parts, the MPNc, and the lateral and medial parts of the MPN (Paxinos and Watson, 2014), while the MPN of mice is separated into lateral and medial parts and does not contain a MPNc (Paxinos and Franklin, 2013). Moreover, it was reported that MPA neurons in male mice have more stubby spines than other types of spines (Jean et al., 2017). However, our present study showed that MPN neurons in male rats have more mushroom spines compared with other spines. The structural difference in the MPN between rats and mice may be responsible for the species differences in MPN function. Therefore, there may be species differences in the MPN mechanism that regulates male sexual behavior, although the MPN in both rats and mice is essential for the regulation of male sexual behavior.

As the result of sexual behavior tests in experiment 1, we divided male rats into two groups. Ejaculators, males having successful copulation 2–3 times, and non-ejaculators, males that displayed mounting and intromission against estrous females but were not able to ejaculate. Obviously, the difference between the non-ejaculators and ejaculators was the presence or absence of ejaculation. Moreover, the ejaculators displayed more mounts and intromissions than the non-ejaculators. A greater number of mounts and intromissions presumably leads to ejaculation in the ejaculators. Additionally, the current study may indicate the dendrite diameter of MPNlm neurons in the ejaculators is larger than that in the non-ejaculators, because the surface area of dendrites in MPNlm neurons were significantly greater in the ejaculators than in the non-ejaculators. According to cable theory, excitatory postsynaptic current is carried a longer distance within a dendrite when the dendrite is thicker, which increases the probability of neuronal excitation. Understanding the differences in sexual behavior performance between the two groups requires further investigation. However, the structural difference in dendrites of the MPNlm may be related to the differences in sexual behavior performance between the non-ejaculators and ejaculators. The differences in behavior performance did not result from differences in blood testosterone levels, because there was no significant difference in serum testosterone concentrations among sexually naive males, and non-ejaculators and ejaculators.

5. Conclusion

Repeated copulatory experience, specifically ejaculation at least twice, significantly reduced the density of mushroom spines in the MPNc, but not in the MPNlm, of male rats. Furthermore, mRNA levels of the actin-binding proteins, drebrin A and spinophilin, in the MPNc, but not in the MPNlm, significantly decreased after male rats had ejaculated for their first and second times. Additionally, the current study showed that the mRNA levels of drebrin A and spinophilin in the MPNc are not changed by stimuli from mounts and intromissions. These results suggest that copulatory experience with ejaculation reduces mushroom spine number in the MPNc, but not in the MPNlm, which is partly due to downregulation of genes encoding acting-binding proteins. The region-specific effects of copulatory experience with ejaculation on synaptic plasticity may alter the function of the MPNc to regulate sexual behavior in male rats.

Author contributions

S. N., M. M., K. U., and S.T. performed the experiments and analyzed data. S. T. designed the study and wrote the paper.

Disclosure

The authors have no conflicts of interest to disclose.

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