Evaluation of changes in anxiety, depression, social behavior, and oxytocin mRNA levels in adults after adolescent interventions in maternal-separated mice

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Abstract

It has been reported that the oxytocin-mediated signal transduction system is biologically altered by traumatic childhood experiences. Oxytocin has also been shown to be involved in stress, social behavior and attachment with others. The effects of oxytocin administration on abused children according to their developmental stage and the changes in the oxytocin signaling pathway due to oxytocin administration or attachment formation are largely unknown. In this study, we used maternal-separated (postnatal day［PD］2-15) mice as a model for childhood adversity and investigated the effects of intervention during adolescence(PD 33-47) by continuously administering oxytocin(1mg/kg/day) or cohabitating them with littermates who had not experienced maternal separation. Cohabitation group was created to develop healthy relationship and attachment with other individuals. The effects of interventions on anxiety- and depression-associated behavior and social behavior in adult mice(N=57) were assessed using behavioral assays from PD60 to PD65. Oxytocin signaling was evaluated by quantification of *oxytocin(OXT)* and *oxytocin receptor(OXTR)* mRNA in the hypothalamus using RT-PCR. Results of our experimental separation and cohabitation protocols showed that the maternal-separated mice

exhibited improved social behavior in social interaction test through each intervention. Biological analysis showed no significant changes in *OXT* and *OXTR* mRNA expression in the hypothalamus. Obtained result showed that oxytocin administration and cohabitation with healthy littermates to maternal-separated mice in adolescent improved social behavior in adulthood. However, the result of *OXT* and *OXTR* mRNA expression in the hypothalamus did not reveal significant changes through each intervention.

Key words

Oxytocin, Adolescence, Social behavior, *Oxytocin* mRNA

Footnotes

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Introduction

Adversity experiences in early childhood are known to be a risk for a variety of mental illnesses, and many studies have shown an association with post-adult anxiety disorders, depression, substance abuse disorders, and personality disorders [1)-3)]. Reports also suggest that incompetent child care is associated with attachment anxiety and attachment avoidance in adult[4)]. Alterations in oxytocin-mediated signaling systems have been reported as a biological result of childhood trauma[5)]. Oxytocin is a hormone synthesized in the hypothalamus and is widely known for its effects on uterine contractions and ejaculation. In the medical field, it has been used as a drug to induce labor and delivery. Recently, its involvement in social behavior, such as parent-child attachment behavior, has attracted attention[6)]. In healthy rats, oxytocin administered continuously before and after weaning and during adolescence has been reported to result in improved social behavior, decreased anxious behavior, and increased *oxytocin receptor* mRNA levels in the hypothalamus after adulthood [7)]. Maternal separation is a frequently used model for childhood adversity in rodents [8)]. Although there is a report [9)] that pre- and post-weaning administration of oxytocin to rats with maternal separation improved social behavior and, reduced

anxious behavior, changes in the oxytocin system across different brain regions and changes in behavior depending on the timing of administration are largely unclear. In addition, when considering its application to actual clinical practice, it is thought to be difficult to administer oxytocin continuously on an outpatient basis to abused young children. In addition, the oxytocin system has been reported to be highly plasticized during the early postnatal period and adolescence[10]. Thus, a clear need for understanding the effects and mechanism of action of oxytocin administration during adolescence. On the other hand, as an environmental intervention, it has been reported that children with attachment disorders due to child abuse reduced interpersonal problems and attachment avoidance and improved social behavior when they form attachments with others in a safe environment[11]. We assumed that oxytocin is involved in the mechanism of this improvement, however, changes in oxytocin signaling pathways in various brain regions are unknown. In this study, cohabitation was done to develop healthy relationship and attachment with other individuals. We continuously administered oxytocin or cohabitated maternal-separated mice with littermates during adolescence. Thus, we investigated the effects of

interventions, on anxiety, depression and social behaviors as well as the changes in biological mechanisms.

Material &Methods

1, Animals

Pregnant C57BL/6J female mice were purchased from Charles River Laboratories Japan, Inc. Among these mice, 11 pregnant female mice were used for the experiment. One week after the pregnant mice arrived, they gave birth to 57 pups, which were used in the experiment. Food and water were available ad libitum. The vivarium was maintained at 21±1 ℃. Room lights were switched to light/dark every 12 h （lit at 8:00 am. The research protocol was approved by the Fukuoka University Ethical Review Board on Animal Testing (approval number: 2106022).

2, Experimental design

The procedure for separation of mothers and pups was performed according to previous studies [12]. They were divided into two groups based on their childhood environments. Twenty of the 57 animals (nine females and 11 males) were raised as the standard nest (SN), and 37 of the 57 (nineteen females and 18

males) were raised as the maternal separate (MS). In MS, mothers and infants were separated for 4 h per day from postnatal day (PD)2 to PD5, and for 8 h per day from PD5 to PD16. When separating mothers from their pups, to reduce the psychological burden on the mothers, only the mothers were moved to another gauge first; then, the pups were moved to the gauge for separation, and the mothers were returned to their original gauge. To increase the stress load of maternal separation, early weaning was implemented from PD17 in MS, whereas in SN, normal weaning was implemented at PD21. In SN, the mother and pups were separated at PD21. In the MS, after early weaning, pups were transferred to individual gauges for rearing. Twelve of the 37 mice cohabitated with their siblings growing in the SN. Subsequently, the adolescent PD35 to PD48 pups underwent two interventions: continuous intraperitoneal administration of saline and intraperitoneal administration of oxytocin. Five groups standard nest-normal saline (SN-NS), maternal separate-normal saline (MS-NS), standard nest-oxytocin (SN-OXT), maternal separate-oxytocin (MS-OXT) and maternal separate-cohabitation with healthy littermates (MS-COHAB) were created according to childhood environment and type of injection or cohabitation. Behavioral analysis was conducted after PD60. After behavioral analysis, the

mice were promptly decapitated, and their brains were removed and cryopreserved. Brain sections were then cut, RNA was extracted from the hypothalamus, and RT-PCR was performed. The schedule of the experiment is shown in Figure 1.

3, Drugs

Oxytocin was purchased from MERCK (Darmstadt, Germany). Oxytocin was dissolved in saline and administered intraperitoneally to the mice at a concentration of 1 mg/kg. Oxytocin concentration was determined based on a previous experiment [7].

4, Behavioral Assays

Open Field Test (OFT)

The OFT was used to measure general activity. This procedure was based on a previous experiment [13]. The mouse was placed in the center of the apparatus (30 cm ×30 cm ×40 cm) and allowed to move freely for 5 min. The total distance traveled in the center was analyzed using the Panlab Smart ver 3.0.06.

Elevated Plus Maze Test (EPM)

The EPM was used to measure anxiety-associated behaviors. The procedure was based on a previous experiment [14]. The device consisted of an open arm (50×10

cm), a closed arm with a wall (50×10×40 cm), and a central square (10×10 cm) at a height of 60 cm. The time spent in the open arm was analyzed using the Panlab Smart ver 3.0.06.

Tail Suspension Test (TST)

The TST was used to measure depression-associated behavior. This procedure was based on a previous experiment [15]. The tail of the mouse was fixed to a rod installed in the test device so the mouse was hung upside-down. The immobility time was measured and analyzed using Panlab Smart ver 3.0.06.

Social Interaction Test (SIT)

The SIT was used to measure social behavior in terms of social proximity in mice. This procedure was based on a previous experiment [16]. First, the mice were placed in an open field (30 × 30 × 40 cm) with empty social interaction test cages and allowed to explore freely for 5 min. For the next 5 min, mice were allowed to explore freely in an open field (30 × 30 × 40 cm) containing social interaction test cages, including an unfamiliar mouse. The area close to the social interaction test cages was designated as high interaction zone and the area away from the cages as low interaction zone. The movements of the mice during

the first and the next 5 min were compared and analyzed using the Panlab Smart ver 3.0.06.

5, Biological analysis

RNA extraction and Real-time PCR

After behavioral testing, brain samples were removed and immediately frozen at -80 degrees and the hypothalamus was extracted from frozen brains at temperatures below freezing for RNA extraction. We determined to measure OXT and OXTR mRNA levels in the hypothalamus. The brain areas where the oxytocin system is evaluated generally include hypothalamus, nucleus accumbens (NAc), amygdala (AMY), hippocampus(Hip) , prefrontal cortex(PFC), etc and the hypothalamus was selected in this study because it is the area where changes in oxytocin activity and *OXTR* mRNA were observed after MS or oxytocin administration on previous literature[7)17)] . Total RNA, excluding genomic DNA (gDNA), was extracted from separated brain samples using NucleoSpin® RNA (MACHEREY-NAGEL, Duren, Germany). Total RNA was reverse-transcribed to cDNA using SuperScript IV reverse transcriptase (Thermo Fisher Scientific, MA, USA). All samples were then amplified using THUNDERBIRD® SYBR® qPCR Mix (TOYOBO, Osaka, Japan) in a reaction volume of 20 μl. Referring

to a previous study [18)19)], *oxytocin (OXT)* and *oxytocin receptor (OXTR)* DNA were selectively amplified using each primer, and the *OXT* and *OXTR* DNA expression levels in the hypothalamus of each group were determined using *Actin beta (ACTB)* as an intrinsic control. All reactions were performed according to the manufacturer's protocol.

6, Statistical analysis

Analysis of variance (ANOVA) and Tukey-Kramer's honest significant difference (HSD) tests were used to compare the groups. Statistical significance was set at $P<0.05$. All statistical analyses were performed using JMP 12.2.0 (SAS Institute Inc. Cary, NC).

Results

Open Field Test (OFT)

Analysis of variance for the total distance traveled showed a significant difference ($P<0.01$). MS-OXT showed a significant increase compared to MS-

COHAB ($P<0.01$). MS-COHAB showed a significant decrease compared to MS-NS ($P<0.01$). (Figure 2A)

SN-OXT showed no significant differences compared to SN-NS ($P=0.1813$). (Figure 2B)

Elevated plus maze test (EPM)

Analysis of variance for the rate of time spent in the open arms showed no significant difference ($P=0.26$). (Figure 3)

Tail Suspension Test (TST)

Analysis of variance for the rate of immobility time showed no significant difference ($P=0.84$). (Figure 4)

Social Interaction Test (SIT)

Significant differences were found in the analysis of variance for the rate of change in the low interaction zone stay ratio ($P<0.01$). MS-NS showed a significant increase compared to SN-NS, MS-OXT and MS-COHAB ($P<0.01$, $P=0.0292$, $P<0.01$). (Figure 5A)

SN-OXT showed no significant differences compared to SN-NS ($P=0.9217$). (Figure 5B)

RT-PCR

In the comparison of *OXT* mRNA expression in the hypothalamus with *ACTB* as an endogenous control, a significant difference was found in the analysis of variance ($P<0.01$). MS-NS, MS-OXT, MS-COHAB, and SN-OXT showed significant decreases compared with SN-NS($P=0.0284$, <0.01, 0.0144, <0.01). MS-OXT showed no significant difference compared with MS-NS ($P=0.84$). MS-COHAB showed no significant difference compared to MS-NS ($P=0.10$). (Figure 6A)

In the comparison of *OXTR* mRNA expression in the hypothalamus with *ACTB* as an endogenous control, no significant difference was observed in the analysis of variance ($P=0.87$). (Figure 6B)

Discussion

This study suggested that the administration of oxytocin and cohabitation with healthy littermates during adolescent to maternal-separated mice improved social behavior in the adult stage. The SIT showed a significant increase in MS-NS relative to SN-NS with respect to rate of change in the low interaction zone stay ratio. This indicates that maternal separation in the juvenile stage of the mice resulted in reduced social behavior in the adult stage, which is similar to

earlier literature [20]. The SIT showed a significant difference between MS-NS and MS-OXT, and showed no significance between MS-OXT and SN-NS. This elucidated that oxytocin administration during adolescent improves adult socialization in maternal-separated mice. The SIT showed a significant difference between MS-NS and MS-COHAB, and showed no significance between MS-COHAB and SN-NS. This indicates that cohabitation with healthy mice improved social behavior.

The result of OFT showed significant differences between MS-NS and MS-COHAB, however, no conclusions could be drawn because there was no significant difference between SN-NS and MS-NS, or SN-NS and MS-COHAB. The OFT also showed significant differences between MS-OXT and MS-COHAB, however, it was not possible to conclude because there was no significant difference between SN-NS and MS-NS, and both groups were not significantly different from SN-NS. The results of the OFT showed movement is recognized in all individuals. We confirmed that no individuals were unable to move by the burden of maternal separation. In OFT, EPM, and TST, there was no significant difference between SN-NS and MS-NS. This result differs from those of previous studies on rodents [13]. In order to increase the stress burden

because of maternal separation, early weaning was also performed, similar to the highest stress burden according to previous literature [12]. However, no significant differences in anxiety- or depression-associated behaviors were observed. This suggests the possibility that detailed rearing conditions not described in the literature, such as the time of experiment and the order of behavioral analysis, may affect the results in mice compared to rats, and the results were similar to those of previous systematic reviews with different results for rats and mice [21].

In our biological analysis, a comparison of mRNA expression of *OXT* in the hypothalamus with *ACBT* as an intrinsic control showed a significant decrease in MS-NS compared to SN-NS, similar to results of existing literature [22]. Although the biological mechanism of the sociability changes induced by drug administration and environmental intervention was of interest, the biological analysis did not reveal significant changes in *OXT* and *OXTR* mRNA in the hypothalamus in either group, living with siblings and receiving oxytocin, and the mechanism could not be elucidated. There is literature indicating that there are sex differences in changes in the oxytocin system in the brain after maternal separation[23]. In this study, there were not enough individuals to separate the group by sex, and there is a possibility of not separated by sex affected the

biological results. Further studies including larger-scale sex-specific experiments and biological comparisons in other brain regions, such as the AMY, PFC, Hip and NAc with reference to previous literature are needed[24)25)].

MS-COHAB started cohabitation with healthy littermates after weaning not adolescent in order to avoid a decrease in affinity due to temporary separation with siblings. Therefore, it is possible that a significant difference was tend to occur compared to MS-OXT, in which intervention was performed only during adolescent. Several mice died because of the burden of maternal separation, which may have affected the results of behavioral analysis.

This study showed that oxytocin administration during adolescence and cohabitation with healthy littermates improves social behavior in maternal-separated mice. However, in the biological analysis, no significant differences in the expression of *OT* mRNA or *OTR* mRNA in the hypothalamus by each intervention.

Acknowledgments

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The authors declare no conflict of interest.

Legends for Figures:

Fig. 1:

Abbreviations; postnatal day (PD), standard nest (SN), maternal separate (MS),

normal saline (NS), oxytocin (OXT)

Fig. 2:

Results from OFT. Total distance traveled in 5 minutes.

Data are presented as means ± SEM.

* : $p<0.05$

Abbreviations: standard nest-normal saline (SN-NS), maternal separate-normal

saline (MS-NS), maternal separate-oxytocin (MS-OXT), maternal separate-

cohabitation with healthy littermates (MS-COHAB), standard nest-oxytocin

(SN-OXT)

Fig. 3:

Results from EPM. Rate of time spent in the open arms in 5 minutes.

Data are presented as means ± SEM.

* : $p<0.05$

Abbreviations: standard nest-normal saline (SN-NS), maternal separate-normal

saline (MS-NS), standard nest-oxytocin (SN-OXT), maternal separate-oxytocin

(MS-OXT), maternal separate-cohabitation with healthy littermates (MS-

COHAB)

Fig. 4:

Results from a 5 min TST. Rate of immobility time in 5 minutes.

Data are presented as means ± SEM.

* : $p<0.05$

Abbreviations: standard nest-normal saline (SN-NS), maternal separate-normal

saline (MS-NS), standard nest-oxytocin (SN-OXT), maternal separate-oxytocin

(MS-OXT), maternal separate-cohabitation with healthy littermates (MS-

COHAB)

Fig. 5:

Results from SIT. Rate of change in the low interaction zone stay ratio

with an unfamiliar mouse in the social interaction cage relative to that without

the mouse.

Data are presented as means±SEM.

* : $p<0.05$

Abbreviations: standard nest-normal saline (SN-NS), maternal separate-normal saline (MS-NS), maternal separate-oxytocin (MS-OXT), maternal separate-cohabitation with healthy littermates (MS-COHAB), standard nest-oxytocin (SN-OXT)

Fig. 6:

Results from RT-PCR. A: Relative expression level of *OXT* to *ACTB*, B: Relative expression level of *OXTR* to *ACTB*.

Data are presented as means±SEM.

* : $p<0.05$

Abbreviations: standard nest-normal saline (SN-NS), maternal separate-normal saline (MS-NS), standard nest-oxytocin (SN-OXT), maternal separate-oxytocin (MS-OXT), maternal separate-cohabitation with healthy littermates (MS-COHAB)

Figure. 1

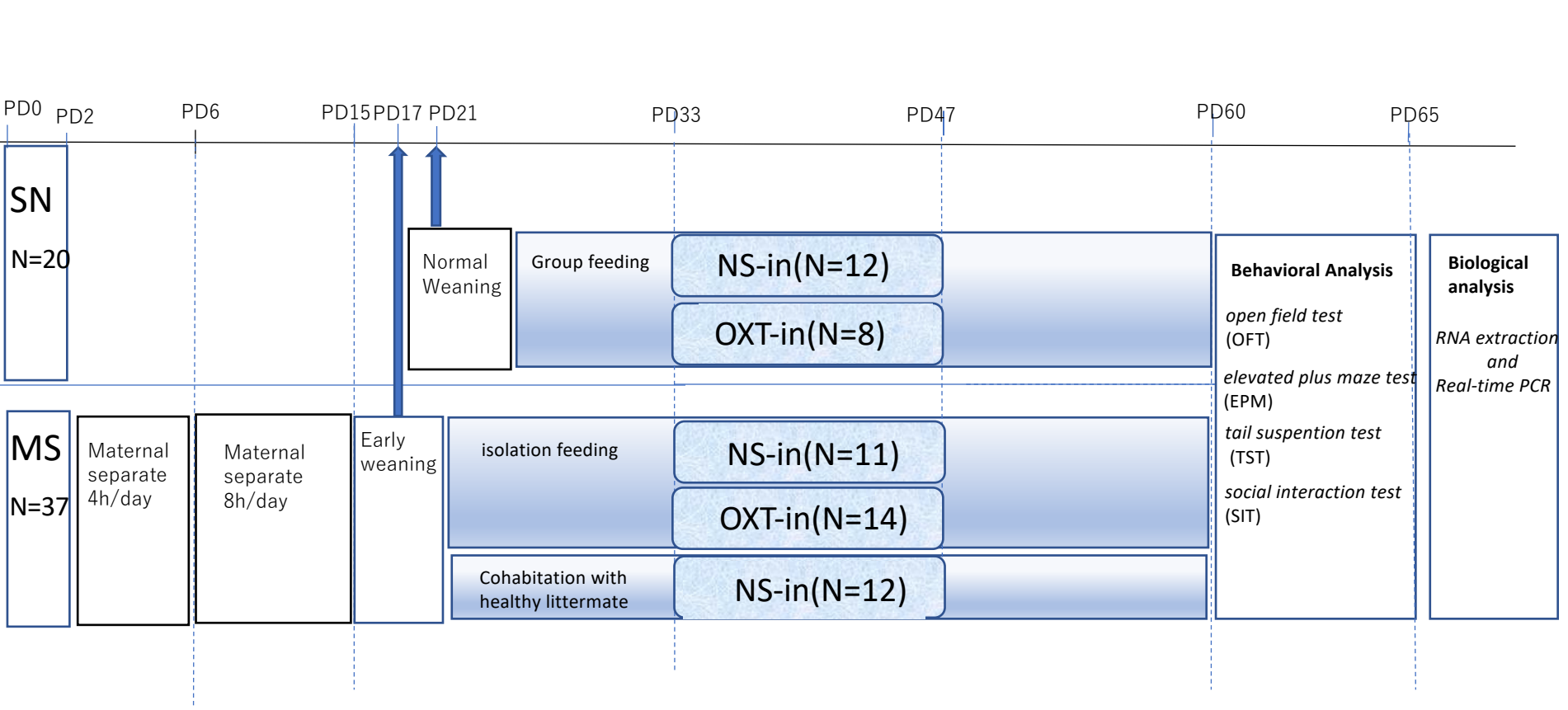

Experimental design
PD0
PD2
PD6
PD15
PD17
PD21
PD33
PD47
PD60
PD65
SN
N=20
Normal Weaning
Group feeding
NS-in(N=12)
OXT-in(N=8)
MS
N=37
Maternal separate 4h/day
Maternal separate 8h/day
Early weaning
isolation feeding
NS-in(N=11)
OXT-in(N=14)
Cohabitation with healthy littermate
NS-in(N=12)
Behavioral Analysis
open field test (OFT)
elevated plus maze test (EPM)
tail suspention test (TST)
social interaction test (SIT)
Biological analysis
RNA extraction and Real-time PCR

Fig. 2 OFT

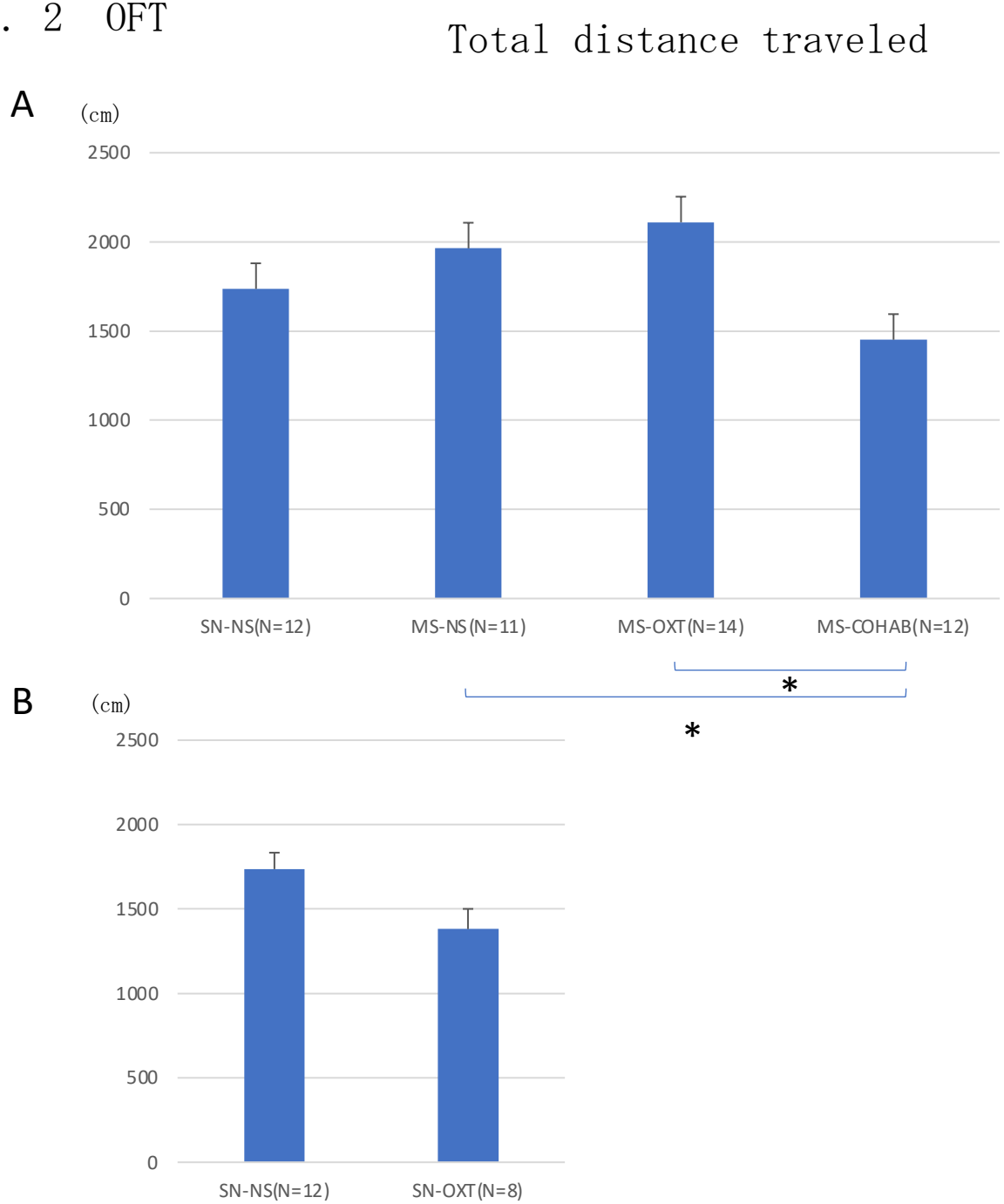

Total distance traveled
A
(cm)
2500
2000
1500
1000
500
0
SN-NS(N=12)
MS-NS(N=11)
MS-OXT(N=14)
MS-COHAB(N=12)
*
*
B
(cm)
2500
2000
1500
1000
500
0
SN-NS(N=12)
SN-OXT(N=8)

Fig. 3 EPM

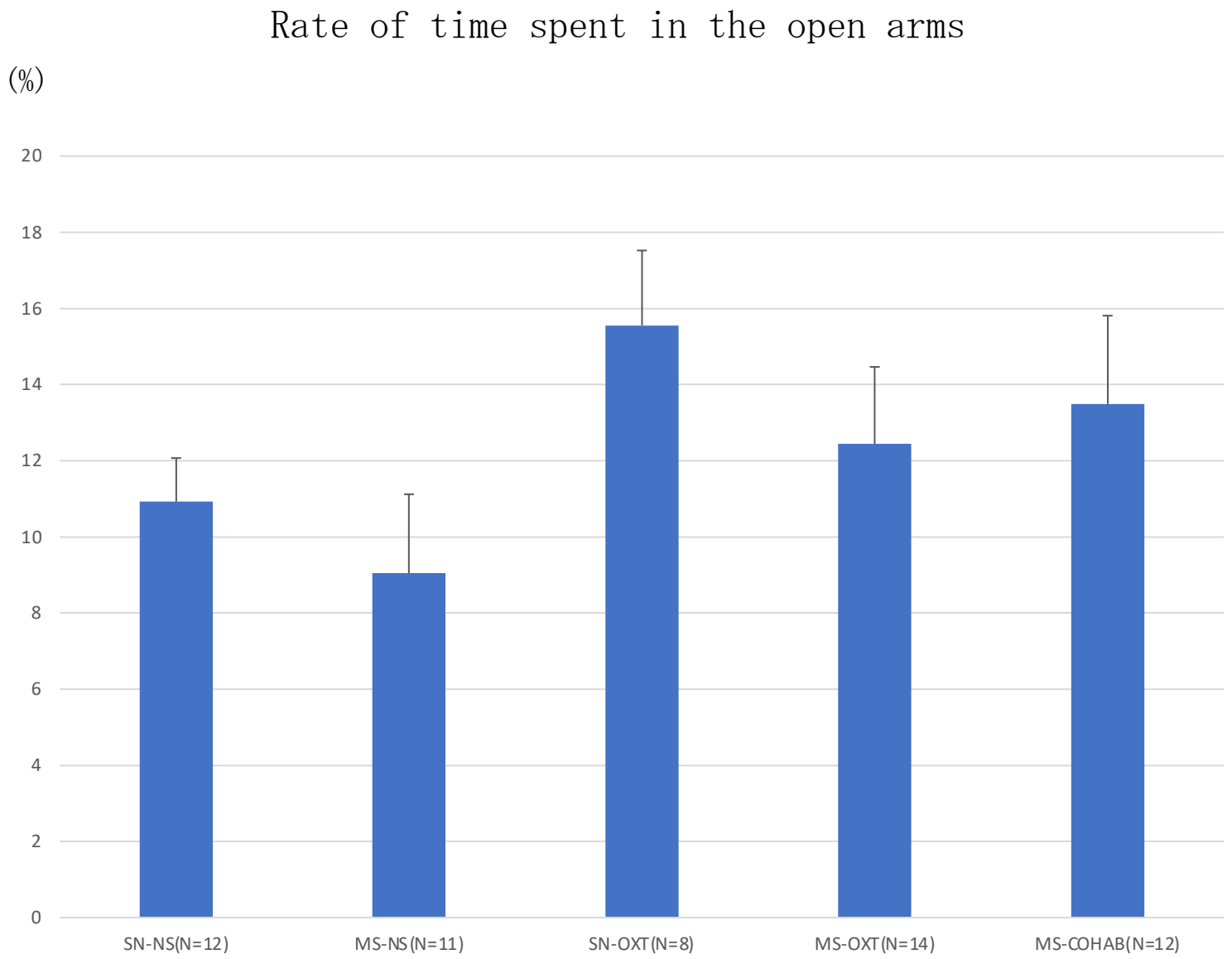

Rate of time spent in the open arms
(%)
20
18
16
14
12
10
8
6
4
2
0
SN-NS(N=12)
MS-NS(N=11)
SN-OXT(N=8)
MS-OXT(N=14)
MS-COHAB(N=12)

Fig. 4 TST

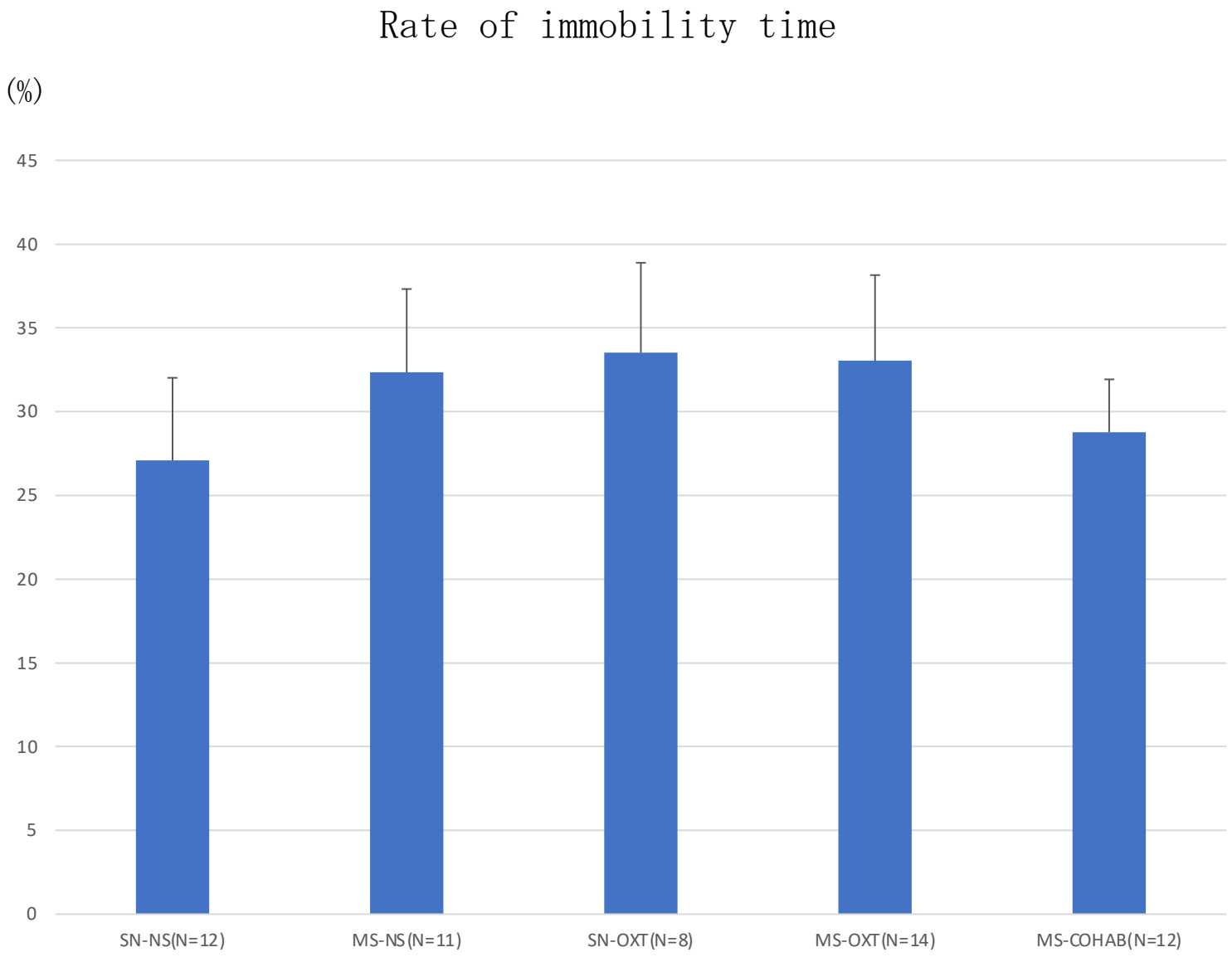

Rate of immobility time
(%)
45
40
35
30
25
20
15
10
5
0
SN-NS(N=12)
MS-NS(N=11)
SN-OXT(N=8)
MS-OXT(N=14)
MS-COHAB(N=12)

Fig. 5 SIT Rate of change in the low interaction zone stay ratio

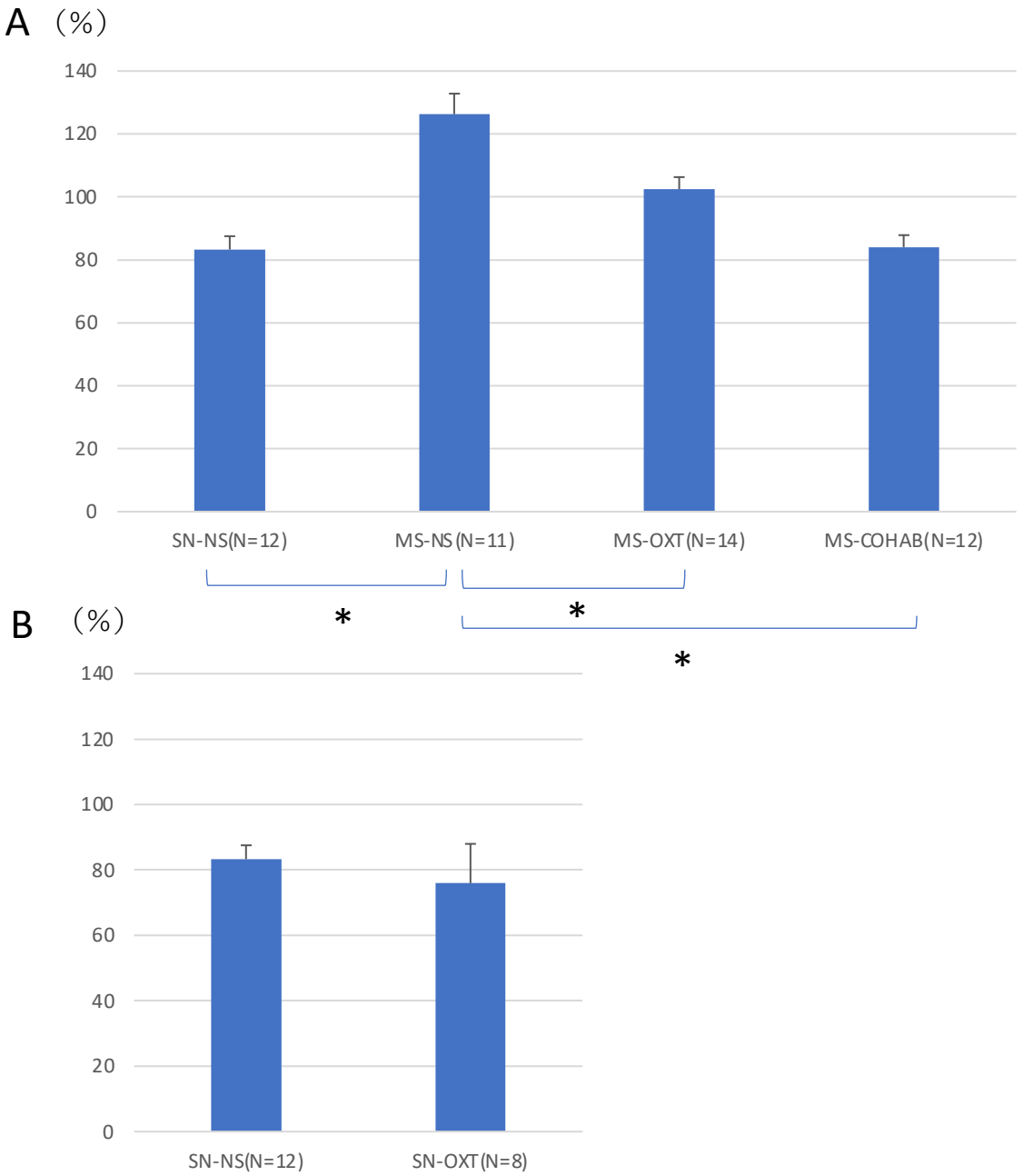
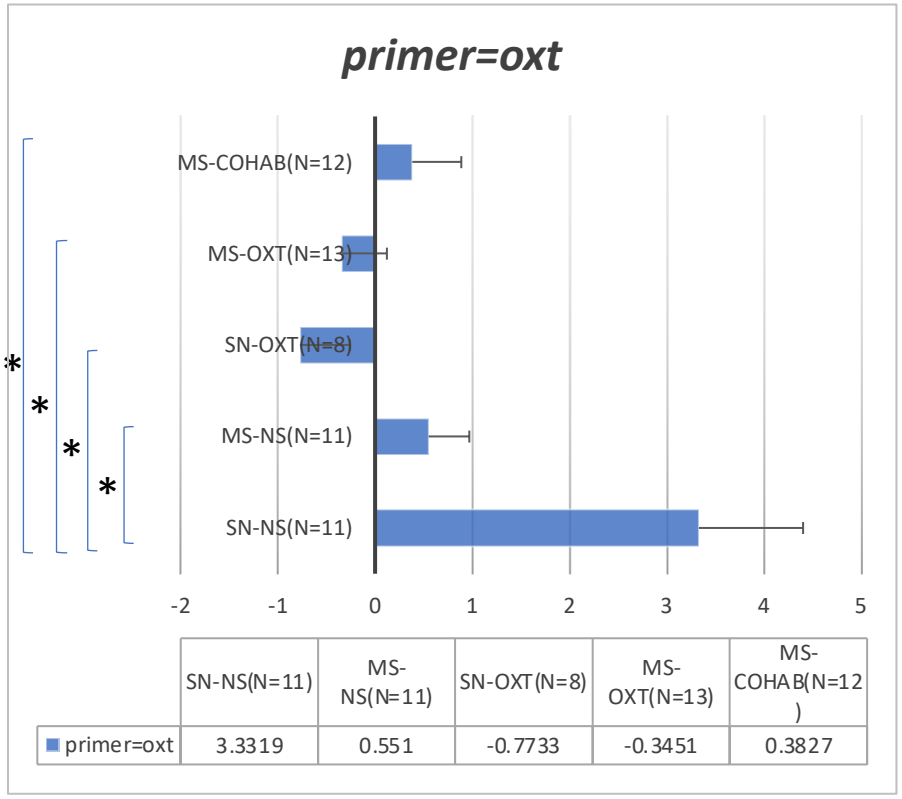


Fig. 6

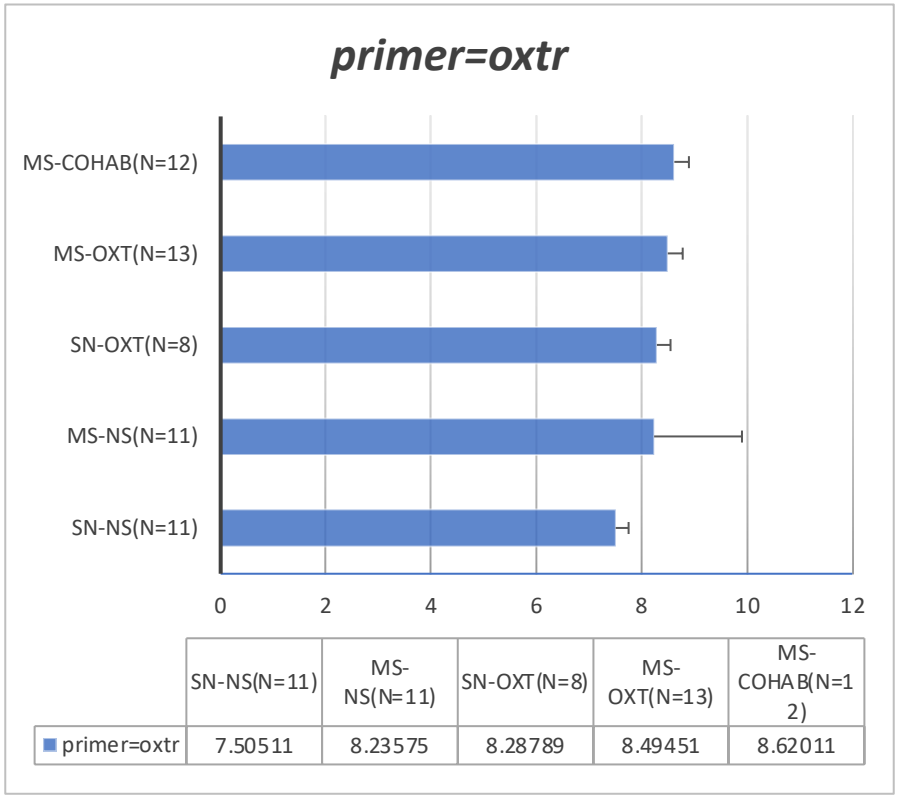
Relative expression level of *OXT* and *OXTR* to *ACBT*

A



	SN-NS(N=11)	MS-NS(N=11)	SN-OXT(N=8)	MS-OXT(N=13)	MS-COHAB(N=12)
primer=oxt	3.3319	0.551	-0.7733	-0.3451	0.3827

B



	SN-NS(N=11)	MS-NS(N=11)	SN-OXT(N=8)	MS-OXT(N=13)	MS-COHAB(N=12)
primer=oxtr	7.50511	8.23575	8.28789	8.49451	8.62011