Circadian-dependent learning and memory enhancement in nociceptin receptor (NOP)-deficient mice with a novel KUROBOX apparatus using stress-free positive cue task

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Abstract

Using novel apparatus, KUROBOX, learning and memory behaviors as well as various parameters of movement activity were re-evaluated in mice deficient for nociceptin/orphanin FQ receptor (NOP\textsuperscript{−/−} mice) or \(\mu\)-opioid receptor (MOP\textsuperscript{−/−} mice). This method has the advantages that no handling procedures are required throughout the experiments performed over 3 days; positive cue paradigms are used without water or shock stress; and the method does not disturb the nocturnal habit of mice. NOP\textsuperscript{−/−} mice displayed a significant enhancement of learning and memory under stress-free conditions, but there were no changes in the various physical and psychological parameters of movement activity (nest stay ratio, distance moved, speed and angle in the movement) and biological rhythm that were measured. Enhancement of nocturnal learning was observed during the first 12 h dark cycle, and enhancement of memory was observed at the beginning of the second dark cycle, in NOP\textsuperscript{−/−} mice. By contrast, MOP\textsuperscript{−/−} mice showed no significant change in learning and memory behaviors or in physical and psychological parameters of movement activity, except for speed: MOP\textsuperscript{−/−} mice showed a significant decrease in speed of movement. Thus, the KUROBOX apparatus provides a useful alternative method to evaluate learning and memory activity under the more physiological conditions. In addition, this apparatus has an advantage that various physical and psychological parameters of movement activity affecting learning and memory behavior are also evaluated at the same time.
Introduction

The nociceptin/orphanin FQ receptor (NOP) was cloned as an opioid receptor homolog, which is coupled, like the opioid receptor, to G-proteins of the $G_{i/o}$ type (Mollereau et al., 1994). However, it is expressed in a distinct brain site from the opioid receptor, and it is thought that NOP regulates very different physiological functions including an anti-opioid effect (Jenck et al., 1997; Griebel et al., 1999; Ueda et al., 2000). On the one hand, suggesting a possible role in memory and learning, it is known that agonist stimulation of NOP as well as $\mu$-opioid receptor (MOP) impairs spatial learning (Castellano, 1975; Sandin et al., 1997). In experiments with receptor deficient mice, the NOP$^{-/-}$ mice showed a facilitation of long-term potentiation and learning abilities (Manabe et al., 1998), whereas MOP$^{-/-}$ mice showed spatial memory impairment (Jamot et al., 2003; Jang et al., 2003). Memory and learning behaviors are potentially affected by various physical and psychological actions. In fact, it is assumed that the antidepressant phenotype of NOP$^{-/-}$ (Gavioli et al., 2003), and the anti-anxiety and antidepressant phenotype of MOP$^{+/+}$ (Filliol et al., 2000), greatly influence memory and learning behavior. Therefore, it is difficult to truly evaluate the physiological role of NOP and MOP in terms of cognition.

Kurokawa et al. (2003) recently developed a new apparatus called KUROBOX to evaluate the spatial memory and training effect (learning) in mice. The food search task test in KUROBOX apparatus has many advantages. 1) It does not use stressful or negative cues. 2) It can be performed for longer periods without the need for handling and during the nocturnal active phase. Here, we attempted to re-evaluate the physiological role of NOP and MOP in terms of memory and learning behaviors in experiments using NOP$^{-/-}$ and MOP$^{+/+}$ mice under
the stress-free conditions with KUROBOX.
Materials and methods

Animals

We used male wild-type (WT) littermate (Tagawa Experimental Animals, Nagasaki, Japan), NOP\(^{-/-}\) (Nishi et al., 1997) and MOP\(^{-/-}\) (Matthes et al., 1996) mice aged 12-20 weeks and weighing 24~28 g. The original lines of NOP\(^{-/-}\) and MOP\(^{-/-}\) mice were backcrossed to the inbred C57Bl/6 strain for at least 10 generations. One day before testing, the mice were acclimated to individual cages in a temperature-controlled room (22 ± 2ºC) with a 12-h-dark cycle (08:00-20:00 light) and free access to a powdered diet (MF Oriental Yeast Tokyo, Japan) and drinking water. Mice were then moved to the nest of KUROBOX at 4 p.m. for further acclimatization for 4 h. Experiments were performed in accordance with the Guidelines for Animal Experiments of the Nagasaki University.

Design of KUROBOX apparatus

We used the KUROBOX apparatus, which has been previously reported (Kurokawa et al., 2003). In the present study, the KUROBOX was modified slightly in terms of the size of apparatus and the structure of the food station. Briefly, the apparatus is an individual box, in which the locomotion of a mouse is monitored by infrared photo-sensors placed at a height of 18 mm above the floor of stainless-steel mesh. The nest \([84 \times 148 \times 135\text{mm} (\text{Length} \times \text{Width} \times \text{Height})]\) was separated from the square observation field \([230 \times 230 \times 190\text{mm} (\text{Length} \times \text{Width} \times \text{Height})]\) with a partition. The mouse could move freely back and forth across the partition between the nest and the observation field. The barycentric coordinate of the mouse was recorded by each of 32 evenly-spaced infrared photo-sensors (and 32 corresponding...
photo-beams on the opposite side) along the walls (x-axis and y-axis). Food stations were set up at the four corners of the observation field. Each food station consisted of a polyvinylchloride wall unit (30 × 45 × 20 mm), and a replaceable stainless mesh plate with two round holes (each 15 mm in diameter) for accessing the powdered food. The rotary feeder, which was set under the floor, consisted of a disk-holder of powdered food. One quarter of the disk was filled with powdered food. The other three quarters were put with small amounts of powdered food, to which mice cannot reach, in order to even the smell from each food center. In addition, rotary feeder may facilitate the diffusion of smell in the apparatus. The rotary feeder was controlled so as to move and stop just beneath each station. The correct food station changed in a counter-clockwise direction every 4 h (Fig. 1).

Measurement of memory and learning using food search task

The barycentric coordinate of the test mouse was recorded every 1 s. Regions of interest (ROIs) were set at the four corners of the observation field, in which each ROI consisted of a 60 × 60-mm square area. Attempts to visit ROIs were counted when the barycenter of the mouse stayed within an ROI for more than 6 s. To analyze the nocturnal performance of a mouse, we recorded locomotion for 3 days. Here, we evaluated the frequency of total and correct visits, total distance moved, nest stay ratio, movement speed and angle, and diurnal rhythm of movement, all of which were analyzed from the trajectory of movement. The lines of the diurnal rhythm were smoothed using a 3-h moving average with a weighing ratio of 0.2:0.6:0.2. Dark ratio was calculated as the ratio of the distance moved during the dark phase to the distance throughout the whole day.
Statistical analysis

Data are expressed as the mean ± S.E. for each group (n=11). The correct ratio for every 4 h was analyzed by two-way analysis of variance. Total distance, nest stay ratio, moving speed, moving angle and dark ratio were analyzed by one-way analysis of variance. Statistical significance was calculated using Bonferroni’s multiple comparison test.
Results

Enhancement of learning and memory in NOP<sup>±</sup> mice.

The first turn-round was set at 8 p.m., and measurement of trajectory was then started. The time series graphs of visits to the four ROIs by WT and NOP<sup>±</sup> mice are shown in Figs. 2A and 2B, respectively. The frequency of visits to each ROI in 10-min intervals is indicated on a gray scale from white to black, where black indicates that the mouse frequently visits the ROI. As the station supplied with food moves every 4 h, mice tend to move to the ROIs. Throughout experiments with both NOP<sup>±</sup> and WT mice, a higher visit frequency was observed during the dark phase (20:00 ~ 8:00), as expected. However, NOP<sup>±</sup> mice showed a clearer slanting shift of visits to ROIs than WT mice. As there was no significant difference in food-intake activity among these mice either in the initial 1<sup>st</sup> interval of day 1 or dark and light phases throughout experiments for three days (Fig. 3A-C), it is evident that NOP<sup>±</sup> mice have performed more correct visits than the WT. As shown in Fig. 4A, the quantification of the correct ratio [visits to the station with food compared to those to all stations] for every 1 h demonstrated that NOP<sup>±</sup> mice showed higher ratios throughout the 3 days. To statistically compare the difference between NOP<sup>±</sup> and WT mice, we calculated the correct ratio for every 4 h. As shown in Fig. 4B, WT mice showed a significant increase in the correct ratio during the 3<sup>rd</sup> interval (III; 8-12 h) of day 1, compared with the 1<sup>st</sup> interval (I; 0-4 h). NOP<sup>±</sup> mice, on the other hand, showed a slight, but not significant increase during the 1<sup>st</sup> interval, compared with WT mice. However, NOP<sup>±</sup> mice showed a significant increase during the 2<sup>nd</sup>
and 3\textsuperscript{rd} intervals, and they showed a higher correct ratio at the 3\textsuperscript{rd} interval than WT mice. On days 2 and 3, the correct ratios in the 1\textsuperscript{st} interval in WT mice were decreased, compared with the values at 3\textsuperscript{rd} interval of the preceding day. Although these ratios were also decreased in NOP\textsuperscript{-/-} mice, higher ratios were observed in NOP\textsuperscript{-/-} mice than in WT mice throughout day 2 and 3.

**Physical and psychological phenotypes in NOP\textsuperscript{-/-} mice**

There were no significant differences in the total distance moved (m/72 h) and nest stay ratio (\%) between NOP\textsuperscript{-/-} and WT mice (Fig. 5A, 5B). Furthermore there was no significant change in the profile of movement speed, which was calculated from recorded distances moved (cm) in 1s (Fig. 5C). Although the speed of movement was divided into low- (0 ~ 0.9 cm/s) and high-speed (above 0.9 cm/s) classes for statistical comparison, there was still no difference. There was no significant difference in movement angle between these mice in terms of mean (radian), kurtosis (Kw) and skewness (Sk) of Gaussian distribution (Fig. 5D). As the present study mainly evaluates various visit or locomotive parameters during the dark phase, it is important to evaluate the locomotor activity in terms of rhythm. However, there was no significant advance or delay of dark phase activity, or changes in dark ratio in NOP\textsuperscript{-/-} mice (Fig. 5E and inset).

**No memory and learning phenotype in MOP\textsuperscript{-/-} mice**
As mentioned above, the visits to ROIs were monitored for three days using MOP−/− mice. Unlike NOP−/− mice, MOP−/− mice did not show a clear slanting shift of visits to ROIs (Fig. 2C). The quantification of the correct ratio for 1 h or 4 h also demonstrated no change between WT and MOP−/− mice (Fig. 6A, 6B).

**No other phenotypes except movement speed in MOP−/− mice**

There were no significant differences in the total distance moved and nest stay ratio between MOP−/− and WT mice (Fig. 7A, 7B). However, unlike NOP−/− mice, MOP−/− mice show a significantly higher low-speed ratio and, inversely, a lower high-speed ratio, than WT mice (Fig. 7C). As mentioned above, the movement angle is an important parameter, but there was no significant difference between MOP−/− and WT mice in terms of mean, kurtosis (Kw) and skewness (Sk) of Gaussian distribution (Fig. 7D). Although there was a slight delay in the onset of movement during the dark phase, there was no significant change in the dark ratio in MOP−/− mice (Fig. 7E and inset). These data suggest that the MOP has only a slight effect on biological rhythm.
Discussion

The activation of opioid receptor family members, such as MOP and NOP, significantly affects memory and learning behaviors (Castellano, 1975; Sandin et al., 1997). Accumulated findings demonstrated that morphine and nociceptin cause amnesic actions in various tests including the Morris water-maze or step-through passive avoidance tests (Baratti et al., 1984; McNamara and Skelton, 1992; Sandin et al., 1997; Mamiya et al., 1999). However, contradictory results have been observed when the experiments are performed with mice deficient for such receptors. NOP−/− mice showed facilitated memory and learning behavior (Manabe et al., 1998), whereas MOP−/− mice still showed amnesia (Jamot et al., 2003; Jang et al., 2003). Although the mechanisms underlying such inconsistency remain to be determined, some of them may be related to the paradigms used for the assessment of memory and learning. As memory and learning have a nature of associated processes and these tests often use negative cues, such as water or electrical shock stress (Morris, 1984), receptor-mediated psychological effects might cause unexpected influences on such behaviors. The stresses caused by handling and performance during the daytime, in spite of a nocturnal habit, are also supposed to affect learning and memory behaviors. Furthermore, some physical and psychological actions of opioids and nociceptin (Reinscheid et al., 1995; Jenck et al., 1997; Asakawa et al., 1998; Griebel et al., 1999) may further affect such behaviors.

KUROBOX is a novel apparatus for the study of memory and learning using a positive cue without handling for all days (Kurokawa et al., 2003). Thus, the stress is
minimized. Moreover, KUROBOX can simultaneously measure detailed motor functions, such as total distance moved and nest stay ratio, which correspond to the measurement of motivation, movement speed, which corresponds to the measurement of the exercise performance, and movement angle, which corresponds to the equilibrium of locomotor. KUROBOX is originally designed for the evaluation of long-term damage of learning and memory by ischemia or the physiological role of specific molecule by the genetic deletion rather than the evaluation of drug-modified memory learning activity, though we have obtained the evidence for the scopolamine-induced amnesia using the same paradigm (Supplemental Fig. 1).

As mentioned above, WT mice showed a significant increase in the correct ratio during the 3rd interval, compared with the 1st, this indicating the learning activity. NOP<sup>-/-</sup> mice also showed more significant increase, as the interval goes, but the learning activity of the mutant mice was significantly higher than the WT mice at the 2<sup>nd</sup> and 3<sup>rd</sup> intervals. It should be noted that the correct ratios during 1<sup>st</sup> interval of day 2 and 3 were apparently lower than that during the 3<sup>rd</sup> interval of each preceding day. Although the mechanisms remain to be determined, there are some possibilities that the memory of acquired learning is lost after the period of light phase, which shows less locomotive or feeding activity, and that the memory is reset or disturbed to some extent by the switch from less active light phase to highly active dark phase. However, the correct ratios during 1<sup>st</sup> interval of day 2 and 3 were higher in NOP<sup>-/-</sup> mice than in WT mice. These findings may suggest that there is a memory
enhancement in NOP\(^{+/+}\) mice.

This enhancement seems to be specific, since there are no significant changes between WT and NOP\(^{+/+}\) mice in various parameters of movement activity, such as distance moved, nest stay ratio, speed and angle in the movement. Although NOP\(^{+/+}\) mice are expected to have a decreased appetite (Stratford et al., 1997), this phenotype was not significant in terms of distance moved and nest stay ratio. In addition, the learning and memory activity in the present study is unlikely related to the food-intake activity, since there was no significant difference in feeding events among WT, NOP\(^{+/+}\) and MOP\(^{+/+}\) mice, as shown in Fig. 3. On the other hand, there is a report that nociceptin causes a regulation of biological rhythm, such as light-induced phase advances (Allen et al., 1999). However, there was no significant advance or delay of active phase and changes in dark ratio, suggesting that the endogenous of nociceptin system has little effect on biological rhythm under natural conditions with a 12-h-dark cycle.

The present finding that NOP\(^{+/+}\) mice show a facilitation of learning and memory behavior in KUROBOX is consistent with the previous findings of Manabe (1998), who first demonstrated that NOP\(^{+/+}\) mice showed a facilitation of long-term potentiation and learning abilities in the Morris water maze and step-through passive avoidance tests. Thus, it is evident that endogenous nociceptin has an in vivo amnesic role both in the absence and presence of stressful handling and cues.

By contrast, the fact that memory and learning behaviors are unaffected in MOP\(^{+/+}\)
mice might be related to the contradictory findings that both morphine administration and deletion of MOP gene showed amnesic effects in the Morris water-maze test, as above-mentioned (Jamot et al., 2003; Jang et al., 2003). As MOP\(^{-/-}\) mice showed no significant changes in various parameters of movement activity except for speed with KUROBOX, physical or psychological effects of endogenous opioids are unlikely to affect memory and learning behaviors in this new apparatus. Although the mechanisms underlying such a contradiction remain to be determined, they might be related to the inconsistent findings that MOP agonists, like endomorphin-1, cause an impairment of memory in some experiments (Ukai et al., 2000; Ukai et al., 2001b), or improve the disturbance of short-term memory resulting from the cholinergic dysfunction (Ukai et al., 2001a). It is fortunate to show that KUROBOX analysis using MOP\(^{-/-}\) mice reveals the phenotype of hypo-locomotion, as previously reported (Matthes et al., 1996; Tian et al., 1997).

The present study showing learning and memory enhancement in NOP\(^{-/-}\) mice is consistent to the findings in previous reports (Manabe et al., 1998; Mamiya et al., 1999), though some other reports show no effect of peptide NOP antagonist, [Nphe\(^1\)]-nociceptin (1-13)-NH\(_2\) on memory and learning (Redrobe et al., 2000; Sandin et al., 2004). The discrepancy with the results may be explained by some reasons including antagonist-specificity (Calo et al., 1998) and short half-life of the peptide per se. In the future, however, a pure and long-lasting NOP antagonist might represent good candidates for the facilitation of learning and memory.
In conclusion, KUROBOX is a new apparatus to evaluate not only memory and learning behaviors, but also detailed parameters of movement activity. By combination with established assays, it might provide more reliable evidence for amnesic or nootropic effects of developing compounds.
References


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**Footnotes**

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Legends for figures

Fig. 1. Drawing of KUROBOX excluding the roof. The apparatus consists of a roof, nest, observation field, four stations (#0, #1, #2, #3), water drink nozzle, hole for the nozzle, infrared photosensors, and a rotary feeder. The station supplied with food changed in a counter-clockwise direction every 4 h (#0~#1~#2~#3).

Fig. 2. Time series graph of the visits to the four ROIs. (A) three WT mice (B) three NOP<sup>−/−</sup> mice (C) three MOP<sup>−/−</sup> mice. The intensity of the visits to each ROI in 10-min intervals is indicated on a gray scale from white to black, where black indicates that the mice spent a lot of time at that ROI. This graph shows representative data.

Fig. 3. No change in the feeding-activity between WT and mutant mice. Results represent the number of visits to the food center during 1<sup>st</sup> interval of day 1 (A), during total dark (B) and light (C) phase for 3 days. Comparison was performed between WT and NOP<sup>−/−</sup> or WT and MOP<sup>−/−</sup> mice (n=11).

Fig. 4. Enhancement of learning and memory in NOP<sup>−/−</sup> mice. (A) Time-dependent changes in the three dark periods. (B) Changes for every 4 h. Changes in the ratio of the number of visits to the correct ROI to the number of visits to all ROIs (correct ratio). This graph includes data on 11 WT and 11 NOP<sup>−/−</sup> mice. Each data point represents the mean ± S.E.. #p<0.05,
Fig. 5. Physical and psychological phenotypes in NOP$^{-/}$ mice. (A) Total distance moved. (B) Nest stay ratio. (C) Movement speed. (D) Movement angle. (E) Diurnal rhythm of movement. All parameters were analyzed from the trajectory of movement over three days. Data are presented as mean ± S.E. from experiments using 11 mice.

Fig. 6. No learning and memory phenotype in MOP$^{-/}$ mice. (A) Time-dependent changes in the three dark periods. (B) Changes for every 4 h. Changes in the ratio of the number of visits to the correct ROI to the number of visits to all ROIs (correct ratio). This graph includes data on 11 WT and 11 MOP$^{-/}$ mice. Each data point represents the mean±S.E..

Fig. 7. No other phenotypes except movement speed in MOP$^{-/}$ mice (A) Total distance moved. (B) Nest stay ratio. (C) Movement speed. (D) Movement angle. (E) Diurnal rhythm of movement. All parameters were analyzed from the trajectory of movement over three days. Data are presented as mean ± S.E. from experiments using 11 mice. *: p<0.05 compared to WT
Figure 1

Water drink nozzle

Nest

Rotary feeder

Food station
Figure 2

A WT

B NOP-/-

C MOP-/-

Food station

20:00 0:00 4:00 8:00 12:00 16:00 20:00
Figure 3

A  1st interval of day 1

B  Dark phase for 3 days

C  Light phase for 3 days

The number of visits to the food center

WT  NOP^-/-  MOP^-/-
Figure 4

A

Day 1

Day 2

Day 3

Dark phase

Correct ratio

0.2 0.4 0.6 0.8

20:00 0:00 4:00 8:00

B

Day 1

Day 2

Day 3

Dark phase

Correct ratio

0.2 0.4 0.6 0.8

(0-4h) (4-8h) (8-12h)

WT

NOP-/-

* * *
Figure 6

A

day1       day2       day3

Dark phase

Correct ratio

20:00  0:00  4:00  8:00  20:00

WT      MOP

B

day1       day2       day3

Dark phase

Correct ratio

I (0–4h)  II (4–8h)  III (8–12h)

WT      MOP
Supplemental Fig. 1 Scopolamine-induced amnesia in KUROBOX assay

Scopolamine hydrobromide (Sigma, St. Louis, MO) was given to C57BL/6 mice in a dose of 2 or 20 mg/kg s.c. 30 min prior to the start of dark phase of day 1. Correct ratios were evaluated during three intervals of day 1. Data are expressed as the mean ± S.E. for each group (saline n=12, Scopolamine 2 mg/kg n=9, Scopolamine 20 mg/kg n=9). Statistical significance was calculated using Turkey-Kramer multiple comparison test. Other details including comparison between control and scopolamine-treated groups were given in the legend of Fig. 4. #:p<0.05 compared to 1st interval (0~4hr) *:p<0.05 compared to saline at the interval.