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Research Report

Nociceptin/orphanin FQ reverses mecamylamine-induced learning and memory impairment as well as decrease in hippocampal acetylcholine release in the rat

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ABSTRACT

Nociceptin/orphanin FQ is an endogenous neuropeptide that plays important roles in several physiological functions including pain, anxiety, locomotion, learning, and memory. We previously reported that low doses of nociceptin improved the scopolamine-induced impairment of learning and memory in the passive avoidance test and the spontaneous Y-maze alternation task in mice. In the present study, the effects of nociceptin on learning and memory impairment as well as the decrease in acetylcholine release induced by mecamylamine were investigated in rats. Mecamylamine (49 μmol/kg, s.c.), a nicotinic acetylcholine receptor antagonist, impaired learning and memory in the step-through type passive avoidance test and decreased acetylcholine release in the hippocampus, as determined by in vivo microdialysis. The administration of nociceptin (10 fmol/rat, i.c.v.) reversed the impairment of learning and memory and blocked the decrease in acetylcholine release induced by mecamylamine. This ameliorating effect on the mecamylamine-induced impairment of learning and memory was not blocked by [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.), an opioid receptor-like 1 (NOP) receptor antagonist. These results suggest that nociceptin improves the impairment of learning and memory as well as decrease in acetylcholine release induced by mecamylamine, and that these effects may not be mediated by NOP receptors.

1. Introduction

Nociceptin, also known as orphanin FQ, is an endogenous ligand for the opioid receptor-like 1 (NOP) receptor and has some structural homology with the endogenous opioid peptide dynorphin A (1–17) (Meunier et al., 1995; Reinscheid et al., 1995). Nociceptin has important roles in several physiological functions including pain, anxiety, locomotion, learning, and memory. Similarly to dynorphin A, higher doses of nociceptin appear to inhibit synaptic function, although it is not known whether these concentrations are physiologically relevant. For example, nociceptin inhibited voltage-gated Ca^{2+} channels in

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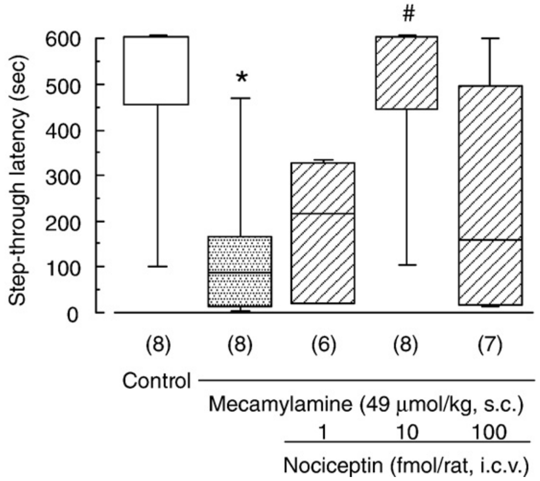

Fig. 1 – Effects of nociceptin on the mecamylamine-induced impairment of learning and memory in a step-through type passive avoidance test. Rats were treated with mecamylamine (49 µmol/kg, s.c.) and nociceptin (1, 10 and 100 fmol/rat, i.c.v.) at 30 and 25 min before the acquisition trial, respectively. The retention trial was carried out 24 h after the acquisition trial. Values show the median (horizontal bar), first and third quartiles (vertical column) and 10th and 90th percentile (vertical lines). The numbers of rats used are shown in parentheses. Significance levels: *$P<0.05$ vs. control (Mann–Whitney's *U*-test), #$P<0.05$ vs. mecamylamine alone (Bonferroni's test).

cultured hippocampal neurons (Knoflach et al., 1996) as well as long-term potentiation in the dentate gyrus and CA1 region of hippocampal slices (Higgins et al., 2002; Yu et al., 1997; Yu and Xie, 1998).

In behavioral studies, intrahippocampal infusion of nociceptin impaired spatial learning in the Morris water-maze task (Sandin et al., 1997, 2004) and intracerebroventricular injection of nociceptin impaired learning and memory in the passive avoidance test (Hiramatsu and Inoue, 1999; Mamiya et al., 1999). Furthermore, Ro 64-6198, a non-peptide NOP receptor agonist, also impaired learning and memory (Higgins et al., 2002). In genetic studies, NOP receptor knockout mice showed facilitated learning and memory as well as and long-term potentiation (Mamiya et al., 2003; Manabe et al., 1998; Taverna et al., 2005). Nociceptin gene knockout mice also showed facilitated learning and memory function (Higgins et al., 2002). These reports indicate that nociceptin and NOP receptors inhibit synaptic plasticity, learning, and memory. Interestingly, we reported that low doses of nociceptin ameliorate the scopolamine-induced impairment of learning and memory in mice (Hiramatsu and Inoue, 2000). However, the mechanisms of this ameliorating effect have not yet been elucidated.

In Alzheimer's disease patients, not only the muscarinic but also the nicotinic receptors are markedly decreased (Nordberg and Winblad, 1986; Quirion et al., 1986; Whitehouse et al., 1986). Scopolamine, a muscarinic acetylcholine receptor antagonist, is widely used to investigate the cholinergic influence on learning ability in experimental animals. However, we previously reported that blockade of nicotinic receptors by mecamylamine also impairs learning ability (Hiramatsu et al., 1998; Hiramatsu and Watanabe, 2006). Nicotinic receptors are localized both on presynaptic axon terminals and at the postsynaptic somatodendritic level (Clarke, 1993; Sargent, 1993). The importance of presynaptic nicotinic receptors was demonstrated in a previous study by McGehee et al. (1995). The sensitivity of presynaptic nicotinic autoreceptors might increase during degeneration of cholinergic neurons as a compensatory mechanism. Although most of the functions of postsynaptic receptors involved in cholinergic signaling in the CNS are not well established (Wonnacott et al., 1989), presynaptic nicotinic receptors on brain cholinergic neurons are known to be tonically active and mediate a positive feedback mechanism that controls cholinergic neuronal activity (Marchi and Raiteri, 1996).

In this study, we therefore investigated the effect of low doses of nociceptin on the impairment of learning and memory and reduction of the acetylcholine release in the hippocampus induced by mecamylamine using a step-through type passive avoidance test and in vivo microdialysis in rats.

2. Results

2.1. *Effects of nociceptin on mecamylamine-induced learning and memory impairment*

Mecamylamine (49 µmol/kg, s.c.) significantly impaired learning when administered 30 min before the acquisition trial (Fig. 1), as reported previously (Hiramatsu et al., 1998; Hiramatsu and Watanabe, 2006). Nociceptin (10 fmol/rat, i.c.v.) administered 25 min before the acquisition trial significantly attenuated the impairment of learning and memory induced by mecamylamine, whereas nociceptin (1 and 100 fmol/rat, i.c.v.) showed no such effect (Fig. 1). No significant differences were observed in responses to electric shocks during the acquisition trial among these groups (Table 1).

Table 1 – Effects of mecamylamine and/or nociceptin on responses to electric shocks in the step-through type passive avoidance test

Treatment	Number (mean ± S.E.M.)		N
	Jump	Vocalization	
Control	0.75 ± 0.25	2.00 ± 0.27	8
Mecamylamine (49 µmol/kg, s.c.)	0.13 ± 0.13	1.38 ± 0.73	8
Mecamylamine + Nociceptin (1 fmol/rat, i.c.v.)	0.17 ± 0.17	0.83 ± 0.54	6
Mecamylamine + Nociceptin (10 fmol/rat, i.c.v.)	0.50 ± 0.19	1.00 ± 0.38	8
Mecamylamine + Nociceptin (100 fmol/rat, i.c.v.)	0.43 ± 0.20	0.57 ± 0.30	7

Rats were treated with mecamylamine (49 µmol/kg, s.c.) and nociceptin (1, 10 and 100 fmol/rat, i.c.v.) 30 and 25 min before the electric shocks, respectively.

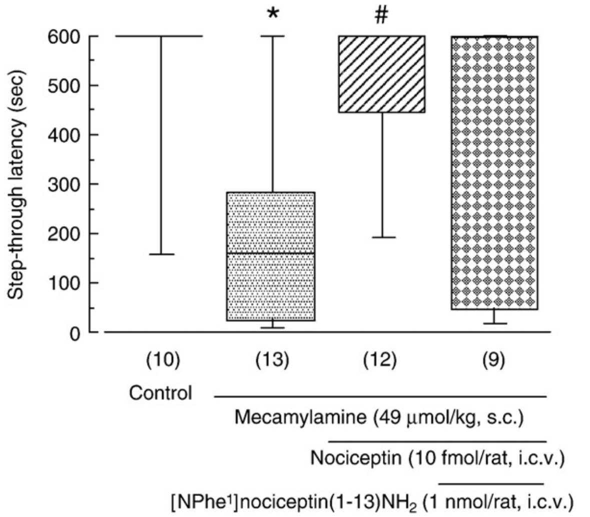

Fig. 2 – Effects of nociceptin and its combination with [NPhe^1]nociceptin(1–13)NH$_2$ on the mecamylamine-induced impairment of learning and memory in the step-through type passive avoidance test. Rats were treated with mecamylamine (49 μmol/kg, s.c.), nociceptin (10 fmol/rat, i.c.v.) and/or [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.) at 30, 25 and 25 min before the acquisition trial, respectively. The numbers of rats used are shown in the parentheses. Significance levels: *$P<0.05$ vs. control (Mann–Whitney's *U*-test), #$P<0.05$ vs. mecamylamine alone (Bonferroni's test).

2.2. *Effects of nociceptin and its combination with [NPhe^1]nociceptin(1–13)NH$_2$ on mecamylamine-induced learning and memory impairment*

To investigate whether the effect of low-dose nociceptin was mediated by NOP receptors, we attempted to block its action using an NOP receptor antagonist, [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.). [NPhe^1]Nociceptin(1–13)NH$_2$ at 1 nmol/rat, which was considered an appropriate dosage for the blockade of the NOP receptor (Fig. 3). However, application of the NOP receptor antagonist did not block the effect of nociceptin on mecamylamine-induced impairment of learning and memory (Fig. 2). There were no significant differences in the responses to electric shocks during the acquisition trial (Table 2).

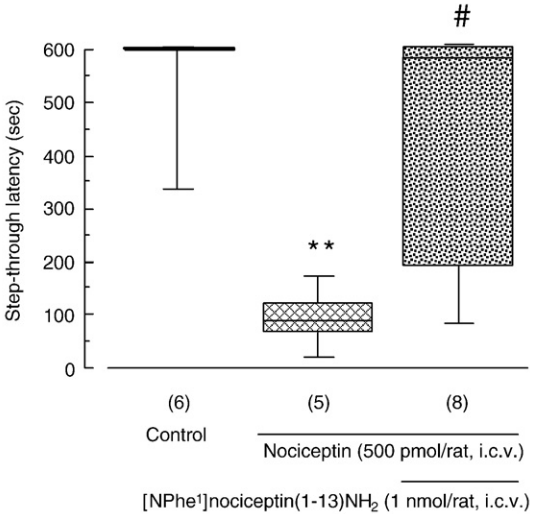

Fig. 3 – Effect of [NPhe^1]nociceptin(1–13)NH$_2$ on impairment of learning and memory in step-through type passive avoidance test induced by high dose of nociceptin. Rats were treated with nociceptin (500 pmol/rat, i.c.v.) and/or [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.) 25 min before the acquisition trial. The numbers of rats used are shown in the parentheses. Significance levels:$P<0.01$ vs. control, #$P<0.05$ vs. nociceptin alone (Mann–Whitney's *U*-test).**

2.3. *Effect of [NPhe^1]nociceptin(1–13)NH$_2$ on induced impairment of learning and memory by high dose of nociceptin*

In accordance with previous reports (Hiramatsu and Inoue, 1999; Mamiya et al., 1999; Sandin et al., 1997), a high dose (500 pmol/rat, i.c.v.) of nociceptin impaired learning and memory in the passive avoidance test (Fig. 3) with a concomitant decrease in the vocalization score (Table 3). This impairment was fully

Table 2 – Effects of mecamylamine, nociceptin and/or [NPhe^1]nociceptin(1–13)NH$_2$ on responses to electric shocks in the step-through type passive avoidance test

Treatment	Number (mean ± S.E.M.)		N
	Jump	Vocalization	
Control	1.00 ± 0.30	1.90 ± 0.31	10
Mecamylamine (49 μmol/kg, s.c.)	0.38 ± 0.21	1.38 ± 0.50	13
Mecamylamine + Nociceptin (10 fmol/rat, i.c.v.)	0.50 ± 0.15	0.75 ± 0.28	12
Mecamylamine + Nociceptin + [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.)	0.44 ± 0.24	0.78 ± 0.32	9

Rats were treated with mecamylamine (49 μmol/kg, s.c.), nociceptin (10 fmol/rat, i.c.v.) and/or [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.) 30, 25 and 25 min before the electric shocks, respectively.

Table 3 – Effects of nociceptin and its combination with [NPhe^1]nociceptin(1–13)NH$_2$ on responses to electric shocks in the step-through type passive avoidance test

Treatment	Number (mean ± S.E.M.)		N
	Jump	Vocalization	
Control	0.67 ± 0.21	2.00 ± 0.37	6
Nociceptin (500 pmol/rat, i.c.v.)	0.20 ± 0.20	0.40 ± 0.40*	5
Nociceptin + [NPhe^1] nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.)	0.88 ± 0.23	0.63 ± 0.18	8

Rats were treated with nociceptin (500 pmol/rat, i.c.v.) and/or [NPhe^1]nociceptin(1–13)NH$_2$ (1 nmol/rat, i.c.v.) 25 min before the electric shocks. Significant levels: *$P<0.05$ vs. control (Mann–Whitney's *U*-test).

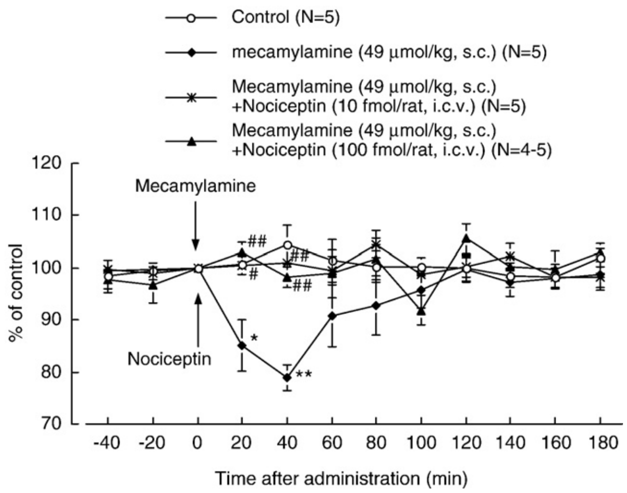

Fig. 4 – Effect of nociceptin on the mecamylamine-induced decrease in acetylcholine release in the rat hippocampus. Mecamylamine (49 μmol/kg, s.c.) was injected at 0 min. Nociceptin (10 fmol/rat, i.c.v.) was injected 5 min after mecamylamine injection. Values represent the means ± S.E.M. of 4–5 rats, as shown in parentheses. Significance levels: *$P < 0.05$, **$P < 0.01$ vs. control (unpaired *t*-test), #$P < 0.05$, ##$P < 0.01$ vs. mecamylamine (Bonferroni's test).

antagonized by [NPhe^1]nociceptin(1–13)NH$_2$ at a dose of 1 nmol/rat without changing the vocalization score.

2.4. *Effect of nociceptin on mecamylamine-induced decrease of acetylcholine release in the rat hippocampus*

Mecamylamine (49 μmol/kg, s.c.) significantly decreased the extracellular levels of acetylcholine in the hippocampus by about 15–20% of base-line levels from 20 to 40 min after injection. Nociceptin (10 and 100 fmol/rat, i.c.v.) completely abolished the mecamylamine-induced decrease of acetylcholine release in the hippocampus (Fig. 4). A low dose of nociceptin alone (100 fmol/rat, i.c.v.) had no effect on the extracellular level of acetylcholine (Fig. 5), whereas a high dose decreased it (Fig. 6).

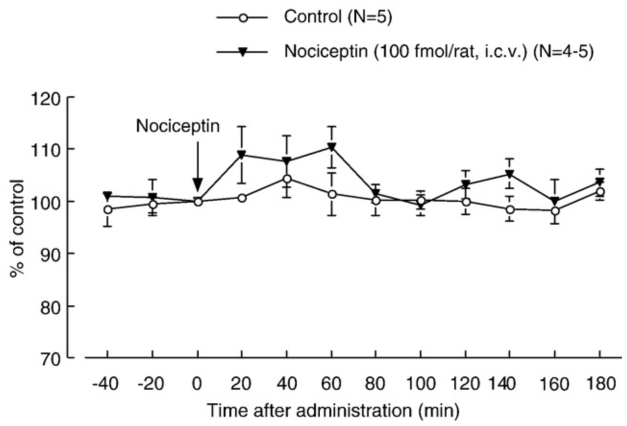

Fig. 5 – Effect of a low dose of nociceptin on acetylcholine release in the rat hippocampus. Nociceptin (100 fmol/rat, i.c.v.) was injected 5 min after saline injection. Values represent the means ± S.E.M. of 4–5 rats, as shown in parentheses.

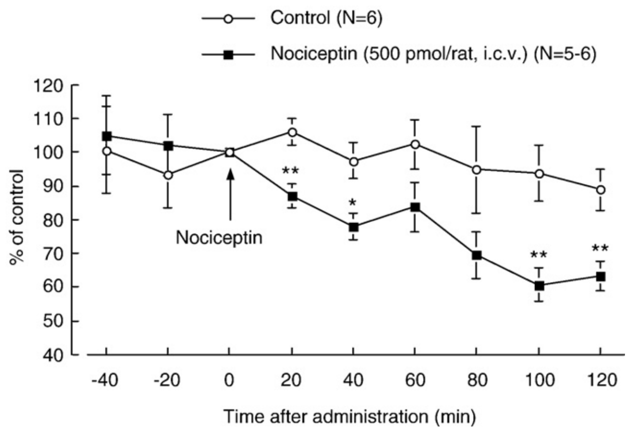

Fig. 6 – Effect of a high dose of nociceptin on acetylcholine release in the rat hippocampus. Nociceptin (500 pmol/rat, i.c.v.) was injected 5 min after saline injection. Values represent the means ± S.E.M. of 5–6 rats, as shown in parentheses. Significance levels: *$P < 0.05$, **$P < 0.01$ vs. control (unpaired *t*-test).

3. Discussion

Nociceptin is an endogenous heptadecapeptide that binds to NOP receptors (Meunier et al., 1995; Reinscheid et al., 1995). The administration of nociceptin caused impairment of learning and memory (Hiramatsu and Inoue, 1999; Mamiya et al., 1999; Sandin et al., 1997) that was blocked by nocistatin, naloxone benzoylhydrazone, [NPhe^1]nociceptin(1–13)NH$_2$ and [Phe$^1\Psi$(CH$_2$–NH)Gly^2]nociceptin (1–13)NH$_2$ (Hiramatsu and Inoue, 1999; Mamiya et al., 1999, 2003; Redrobe et al., 2000; Sandin et al., 2004). Furthermore, a genetic deficit of the nociceptin system facilitated learning and memory function (Higgins et al., 2002; Manabe et al., 1998). These observations indicate that nociceptin and NOP receptors play inhibitory roles in learning and memory. Interestingly, although high doses of nociceptin impair learning and memory, we have demonstrated that low doses of nociceptin ameliorate scopolamine-induced learning and memory impairment in mice (Hiramatsu and Inoue, 1999, 2000). We also confirmed that high dose of nociceptin (5 nmol/mouse, i.c.v.) induced learning and/or memory impairment in the Y-maze and step-down type passive avoidance test with some abnormal behaviors immediately after injection in mice. In present study, a high dose of nociceptin (500 pmol/rat, i.c.v.) significantly decreased the number of vocalizations when rats received electric shocks during the acquisition of passive avoidance, indicating that shock sensitivity had changed. Therefore, we consider that these effects after high doses of nociceptin would be nonphysiological phenomena. The aim of the present study was to examine the mechanism of the effects of low doses of nociceptin. So, we first tested whether low doses of nociceptin had similar behavioral effects on rats and then tested its effect on the reduction in acetylcholine release in the rat hippocampus induced by mecamylamine, a nicotinic acetylcholine receptor antagonist, using a microdialysis technique. In accordance with our previous reports, the present study confirmed that a low dose of nociceptin (10 fmol/rat, i.c.v.) ameliorated the impairment of learning and memory induced by mecamylamine in rats. To clarify whether these effects were mediated by NOP receptors, the NOP receptor antagonist [NPhe^1]nociceptin(1–13)NH_2 was coadministered with nociceptin. However, the low dose effect of nociceptin on mecamylamine-induced learning and memory impairment was not blocked by [NPhe^1]nociceptin(1–13)NH_2 (1 nmol/rat, i.c.v.). To clarify whether the dose of NOP receptor antagonist was appropriate for the blockade of the NOP receptor, we examined the effect of [NPhe^1]nociceptin(1–13)NH_2 on high dose of nociceptin-induced learning and memory impairment. The high dose effect of nociceptin on learning and memory was fully blocked by [NPhe^1]nociceptin (1–13)NH_2 (1 nmol/rat, i.c.v.) without changing the number of vocalizations. These results suggest that high doses of nociceptin impair learning and memory in an NOP receptor-dependent manner, whereas the low dose effects of nociceptin are not mediated by NOP receptors. The present results are consistent with our previous reports showing that low dose effects of nociceptin were not blocked by nocistatin or naloxone benzoylhydrazone (Hiramatsu and Inoue, 2000).

Nociceptin influences pain state, emotional state and food intake (Calo’ et al., 2000). The influence of emotional memory on the passive avoidance test may have affected the present results. In a previous study in mice, we tested spontaneous alternation behavior in the Y-maze, which should not reflect emotional state, or changes in pain or food intake, and we found similar improvements upon nociceptin treatment to those seen in the present study using the passive avoidance test. There are no reports describing emotional behavior on application of the lower doses of nociceptin used in the present study, but we found no effects on the number of vocalizations in response to electric shocks (see Tables).

We have recently reported that dynorphin A (2–13) completely abolished the decrease in extracellular acetylcholine concentration induced by mecamylamine, and this effect was not blocked by nor-binaltorphimine. Based on our previous findings, we hypothesized that dynorphin A (2–13) ameliorates the impairment of learning and/or memory via a non-opioid mechanism by regulating the release of extracellular acetylcholine (Hiramatsu and Watanabe, 2006). Since nociceptin shares high sequence homology with dynorphin A, lacks the N-terminal tyrosine characteristic of opioids (Nothacker et al., 1996), and is expressed in the rat hippocampus, we examined the effects of nociceptin on the extracellular acetylcholine concentration in the rat hippocampus.

Mecamylamine (49 μmol/kg, s.c.) significantly decreased the acetylcholine concentration in the hippocampus as previously reported (Hiramatsu et al., 1998; Hiramatsu and Watanabe, 2006). Interestingly and similarly to dynorphin A (2–13) (Hiramatsu and Watanabe, 2006), low doses of nociceptin (10 and 100 fmol/rat, i.c.v.) prevented the mecamylamine-induced decrease in acetylcholine concentration. Nociceptin itself had no significant effect on acetylcholine release in the rat hippocampus, although 100 fmol nociceptin tended to increase acetylcholine release. These observations led us to speculate that nociceptin ameliorates learning and memory impairment by improving cholinergic malfunction. In the behavioral study, however, 100 fmol nociceptin did not generate significant improvement. The reason for the discrepancy between the microdialysis and behavioral data is not clear. In the microdialysis experiment, only the cholinergic system in the hippocampus was studied because it plays important roles in learning and memory, whereas in the behavioral experiment, other systems or brain areas may have been involved. As seen with other drugs, a bell-shaped dose response was observed in the behavioral experiments.

A high dose of nociceptin inhibited acetylcholine release in the rat striatum, as shown by in vivo microdialysis (Itoh et al., 1999). Basal levels of acetylcholine release in the hippocampus were significantly increased in NOP receptor knockout mice (Uezu et al., 2005), whereas high doses of nociceptin inhibited glutamate release in rat cerebrocortical, cellebellar and brainstem slices (Nicol et al., 1996, 2002). However, Marti et al., (2002) reported that nociceptin application increases extracellular glutamate levels in the substantia nigra pars reticulata using in vivo microdialysis. Thus, nociceptin modulates the levels of several neurotransmitters, including glutamate and acetylcholine, and may thereby ameliorate impairment of learning and memory.

Previous studies examined the mechanisms by which nociceptin impairs learning and memory. Nociceptin inhibited the phosphorylation of calmodulin-dependent protein kinase II in hippocampal slices in mice (Mamiya et al., 2003), the accumulation of cyclic AMP (Mathis et al., 1997), the activation of a voltage-gated Ca^{2+} channel in cultured hippocampal neurons (Knoflach et al., 1996), and long-term potentiation in the dentate gyrus and CA1 region of hippocampal slices (Higgins et al., 2002; Taverna et al., 2005; Yu et al., 1997; Yu and Xie, 1998). These studies showed that these inhibitory effects of nociceptin are involved in the impairment of learning and memory. However, there is as yet no clear explanation of the mechanism of low dose nociceptin-mediated improvement.

Application of Ro 64-6198 mimicked the induction of hypolocomotion by high doses of nociceptin in mice, but it failed to induce hyperlocomotion similar to that produced by low doses of nociceptin (Kuzmin et al., 2004). In Ro 64-6198-unresponsive neurons, nociceptin activated G protein-coupled inwardly rectifying K^+ channels (Chiou et al., 2004). These studies suggest that there is heterogeneity of the NOP receptor. Binding studies with [^{125}I]-Tyr^{14}-nociceptin and [^{3}H] nociceptin revealed curvilinear Scatchard plots that are also suggestive of sites with differing affinity (Mathis et al., 1997; Onali et al., 2001). Therefore, low dose nociceptin-mediated improvement in the present study may be mediated by a subset of NOP receptors or by some mechanism, as it was not blocked by [NPhe^1]nociceptin(1–13)NH_2. Recent studies indicate that cAMP response binding protein (CREB), a transcription factor, and extracellular signal-regulated kinase (ERK), an upstream modulator of CREB, play critical roles in memory formation. Application of nociceptin induced phosphorylation of CREB and ERK in vitro (Kim et al., 2002; Zhang et al., 1999). An alternative possibility in the present study is that the ameliorating effect observed with low doses of nociceptin was mediated through these intracellular signaling pathways. Further characterization of the sites or mechanisms of action of nociceptin may provide new insight into the underlying mechanisms of its excitation and pharmacological role in deficits of cholinergic transmission.

In conclusion, nociceptin showed a biphasic effect on learning and memory. High doses of nociceptin impaired learning and memory function; in contrast, low doses of nociceptin ameliorated the impairment of learning and memory. Although the high-dose effect of nociceptin was mediated by NOP receptors, the mechanism of action of the low-dose effect has not yet been elucidated. Nociceptin abolished the decrease in acetylcholine release in the hippocampus induced by mecamylamine. These studies suggest that nociceptin plays an important role in the modulation of learning and memory function through both NOP receptors and NOP-independent mechanisms, depending on the dosage.

4. Experimental procedures

4.1. *Animals*

Eight-week-old male Sprague–Dawley rats (Japan SLC Inc., Japan) were housed in a room with controlled lighting (12-h light/dark cycle, lights on: 08:00 to 20:00) and temperature (23 ± 2 °C) for at least 3 days before the experiments, and given free access to food and water. Experimental protocols concerning the use of laboratory animals were approved by the animal use committee of Meijo University and followed the guidelines of the Japanese Pharmacological Society [(1992) Guiding Principles for the Care and Use of Laboratory Animals. Folia Pharmacol Jpn 99:35A] and the Interministerial Decree of May 25, 1987 (the Ministry of Education).

4.2. *Surgical procedures*

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Using the coordinates from the stereotaxic atlas of Paxinos and Watson (Paxinos and Watson, 1997), a guide cannula for a microdialysis probe was implanted unilaterally into the hippocampus, and a cannula for drug injection was implanted into the lateral ventricle. The tips of the cannulas were positioned just above the hippocampus (A: −4.1, L: 2.0 V: 3.2 mm from the bregma), and the lateral ventricle (A: −0.8, L: 1.6, V: 4.5 mm from the bregma) of each rat. The animals were allowed to recover from the procedure for 3 to 7 days before the experiment. In the experiment, the dialysis probe (CMA/10) was inserted through the guide cannula and a 3-mm length of dialysis membrane was then advanced into the hippocampus.

4.3. *Passive avoidance test*

One group of rats was trained in a passive avoidance apparatus that consisted of two compartments, one light (25 × 15 × 15 cm high) and one dark, of the same size connected via a guillotine door. On day 1, each rat was placed in the light compartment and then allowed to enter the dark compartment. Rats that had latencies greater than 60 s were discarded as being outside the normal range (pre-acquisition trial). The acquisition trial was carried out 15 min after the pre-acquisition trial. The rat was placed in the light compartment and 30 s later the guillotine door was opened. Once the rat entered the dark compartment, the guillotine door was closed and an electric shock (0.25 mA for 3.0 s) was delivered to the animal via the grid floor. The retention trial was carried out 24 h later. The rat was put in the light compartment and the time taken to enter the dark compartment was recorded (step-through latency). The maximum latency was set at 600 s.

The responses to electric shock were recorded during the acquisition trial. The number of vocalizations and jumps were counted.

4.4. *Microdialysis procedure*

Another group of rats was used for microdialysis experiments. The dialysis probe was perfused with Ringer's solution (composition in mM: NaCl, 147; KCl, 4; $CaCl_2$, 2.3 mM, containing 0.01 mM eserine) at a rate of 2 μl/min and connected to a microinfusion pump (Syringe Infusion Pump 22, Harvard Apparatus, South Natick, MA) by a single-channel liquid swivel. The rat was placed in an individual acrylic cage (30 × 30 × 35 cm high). The dummy cannula was replaced with a dialysis probe and perfusate was collected in 250-μl disposable microcentrifuge tubes secured to the middle of the tether. About 3 h after the probe was inserted, samples (40 μl) were collected at 20-min intervals, and when at least three baseline samples were stable, the drugs were administered. Perfusate samples from the brain were taken up to 120 or 180 min after treatment with drugs or saline. The locations of the dialysis probes were confirmed after the experiments.

4.5. *Analysis of dialysates*

Acetylcholine in the dialysate was quantified by HPLC with an immobilized enzyme column and an electrochemical detector (ECD-300, Eicom Corp., Japan). The mobile phase consisted of 0.1 M sodium phosphate buffer (pH 8.5) containing 1.23 mM sodium 1-decanesulfonate, 593 μM tetramethylammonium chloride and 13.4 μM disodium ethylenediaminetetraacetate, and was delivered by a pump (Intelligent HPLC pump PU-890 or TriRotor V, Japan Spectroscopic Co., Ltd., Japan) at a flow rate of 0.6 ml/min. Aliquots (30 μl) of the dialysate were injected into the HPLC system and separated by a column of Eicompak AC-GEL (4.6 × 150 mm). The enzyme column containing acetylcholinesterase and choline oxidase catalyzed the formation of hydrogen peroxide from acetylcholine and choline. The resultant H_2O_2 was detected by ECD with a platinum electrode at +450 mV vs. Ag/AgCl.

4.6. *Drugs*

The following drugs were used: sodium pentobarbital (Tokyo Chemical Industry Co., Ltd., Japan); mecamylamine hydrochloride (mecamylamine, Sigma); nociceptin (Peptide Institute, Japan); $[NPhe^1]nociceptin(1–13)NH_2$, an NOP receptor antagonist (Tocris). Drugs were dissolved in isotonic saline solution (Otsuka Pharmaceuticals, Inc., Japan).

Mecamylamine was administered (s.c.) 30 min before the acquisition trial of the passive avoidance test or at 0 min in the case of the microdialysis experiment. Nociceptin and [NPhe^1] nociceptin(1–13)NH_2 were administered intracerebroventricularly (i.c.v.) into the lateral ventricle 5 min after mecamylamine injection. These peptides were infused in a volume of 5 μl/rat at a rate of 5 μl/min. Control animals were injected s.c. and i.c.v. with saline. The dose range for nociceptin used in the present was very wide, from 1 fmol to 500,000 fmol, so data are presented using different dose units (fmol and pmol).

4.7. *Data analysis*

The passive avoidance data are shown as the median (horizontal bar), first and third quartiles (vertical column) and 10th and 90th percentile (vertical lines). Significant differences were evaluated using Mann–Whitney's *U*-test for comparisons of two groups and the Kruskal–Wallis non-parametric one-way analysis of variance followed by Bonferroni's test for multiple comparisons. Microdialysis data are shown as means ± S.E.M. of the percentage of the baseline level obtained from each rat before drug administration. Significant differences were evaluated using the unpaired *t*-test for comparisons of two groups and the one-way analysis of variance followed by Bonferroni's test for multiple comparisons. The criterion for significance was $P<0.05$ in all statistical evaluations.

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