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Spinally delivered nociceptin/orphanin FQ reduces flinching behaviour in the rat formalin test

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THE aims of this study were to investigate the dose-dependent effects of spinally delivered nociceptin (0.3, 1, 3.3 and 10 nmol) on flinching behaviour in the rat formalin test and whether these effects were influenced by the concomitant systemic administration of naloxone (3 mg/kg, i.p.). The effect of the highest nociceptin dose differed statistically from vehicle, 0.3 and 1 nmol nociceptin. Following the administration of 1, 3.3 or 10 nmol nociceptin mean total flinches decreased dose-dependently. The effects of 10 nmol nociceptin were not reversed by a high dose of naloxone. We observed a decrease in flinching behaviour with intrathecally to the lumbar enlargement delivered nociceptin and conclude that nociceptin has antinociceptive effects in the rat formalin test.

Key words: Antinociception; Nociceptin; Orphanin FQ; Rat formalin test

Introduction

Nociceptin/orphanin FQ[1,2] is a putative endogenous agonist of opioid receptor-like ORL1 receptors which do not bind classical opioids[3] and are widespread in the rat CNS.[4] Nociceptin is a heptadecapeptide (average M_r 1810)[1] which structurally resembles dynorphin A but does not show appreciable binding to μ, δ or κ receptors.[5] Mice that were injected intracerebroventricularly (i.c.v.) with 55 pmol nociceptin showed decreased latencies to rearing and escape jumping in the hot plate test.[1] Increased reaction times, however, were observed in the hot plate test in mice following i.c.v. administration of 10 nmol nociceptin.[2] This response was interpreted as being due to decreased locomotor activity and decreased muscular tone. When nociceptin was administered intrathecally (2.5–10 nmol) no antinociceptive response was observed in the hot plate test in mice.[2] The same authors found reduced reaction times following the i.c.v. administration of 1, 3 or 10 nmol nociceptin in the tail flick test in mice. These results suggest that nociceptin induces hyper-reactivity to noxious stimuli rather than antinociception. In contrast to these observations, *in vitro* studies demonstrated inhibitory effects of nociceptin in dorsal raphe nucleus neurones of rats[6] and in a human neuroblastoma cell line.[7]

The objectives of the following study were to investigate the effects of spinally delivered nociceptin on flinching behaviour in the rat formalin test, the dose-dependency of these effects and whether these effects are influenced by the concomitant systemic administration of naloxone.

Materials and Methods

Animals: Forty-eight experimentally naive, male Sprague–Dawley rats weighing 350–400 g at the time of surgery were used. Thirty animals participated in the first part and 18 in the second part of the study. The animals had free access to food and water prior to the experiments. They were maintained in climate- and light-controlled rooms (24 ± 0.5°C, 12:12 h dark:light cycle). Each animal was used on one occasion only. In all experiments the ethics guidelines for investigations in conscious animals were obeyed and the procedures were approved by the local Ethics Committee. At the end of the experiment animals were killed by CO_2 inhalation.

Study design: In the first part of the study the flinching behaviour in the formalin test following the randomized and observer-blinded lumbar intrathecal delivery of either 0.3, 1, 3.3 or 10 nmol nociceptin,

or vehicle (saline 0.9%) in 30 rats ($n = 6$ per group) was investigated. In the second part of the study the flinching behaviour in the formalin test following the randomized and observer-blinded administration of either 10 nmol nociceptin by the lumbar intrathecal (i.t.) route and vehicle i.p., vehicle lumbar i.t. + naloxone 3 mg/kg i.p., or 10 nmol nociceptin lumbar i.t. + naloxone 3 mg/kg i.p. ($n = 6$ per group, was investigated.

Nociceptin: Nociceptin was synthetized and quality was assured by the Institute of Biochemistry, University Erlangen-Nüernberg, Germany. Purity was > 95%.

Implantation of lumbar intrathecal catheters: Animals were anaesthetized with ketamine (100 mg/kg i.p.) and xylacine (5 mg/kg i.p.) and implanted with lumbar intrathecal catheters (modified according to Ref. 8). Polyethylene catheters (i.d. 0.28 mm, o.d. 0.61 mm, length 12–13 cm) extended from the cisterna to the rostral edge of the lumbar enlargement. Only rats without relevant disturbances after recovery from anaesthesia were used. Motor function was checked as by monitoring reaction following pinching of the paws and the tail, placing/stepping reflex, and the righting reflex following placement of the rat horizontally with its back on the table. After surgery rats were monitored each day for weight, and for general and neurological well-being.

Formalin test: Intrathecal injection studies were performed 6–10 days after implantation provided that rats showed no disturbances of general well-being, abnormal behaviour, neurological deficits or dysfunction. In the morning of the study day patency of catheters was checked by a saline flush of 10 µl. The formalin test was always performed in the morning in a dedicated room with restriction on sound level and activity. It was carried out in a plexiglas chamber with a mirror mounted at 45° to allow an unobstructed view of the paws. Animals were adapted to the test chambers for at least 15 min prior to the experiments. Treatments were delivered to the lumbar enlargement via the i.t. catheter in a total volume of 10 µl immediately followed by a saline flush of 10 µl. Ten minutes later a formalin test[9] was performed. Formalin (50 µl, 5%) was injected s.c. into the dorsal surface of the left hindpaw. Flinches of the injected paw were counted at 1 min intervals for 60 min. Counts were done by the same observer in all rats. In the second part of the study an additional neurological examination was performed immediately after the formalin test. Rats were then killed and patency (injection of methylene blue solution) and placement of catheters in the subarachnoic space adjacent to the lumbar enlargement of the spinal cord was checked. In the second part of the study naloxone or vehicle was additionally administered i.p. 3 min prior to the formalin injection.

Data analysis: Flinching data of each rat were summarized in 5 min intervals as mean flinches/min and plotted as group means (+ s.e.m.) *vs* time for the first and second part of the study separately (Figs 1,3). Total flinches (sum of all flinches of an individual rat) were plotted *vs* dose or treatment as box plots (Figs 2,4). The box represents the interquartile range, the line within the box the median, the dotted line the arithmetic mean. The ends of the 'whiskers' show maximum and minimum.

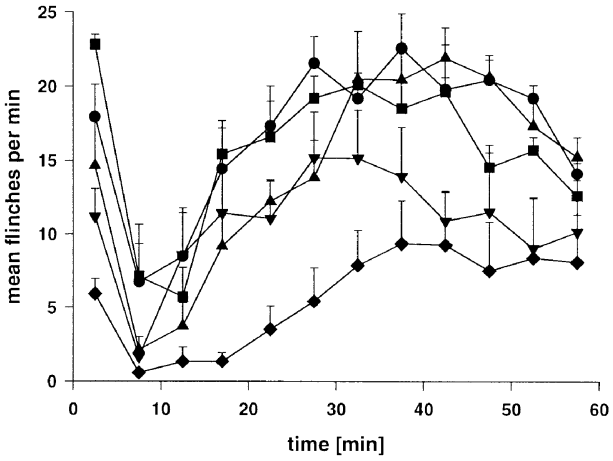

FIG. 1. Time-course of flinching behaviour after the lumbar i.t. administration of either vehicle (■), 0.3 nmol (●), 1 nmol (▲), 3.3 nmol (▼) or 10 nmol (◆) nociceptin which was delivered 10 min before the formalin test ($n = 6$ per group). Data are presented as mean + s.e.m. For graphical clarification flinches are shown as averages/5 min intervals.

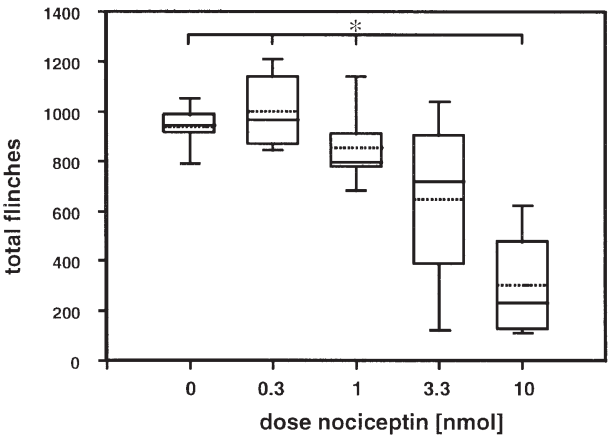

FIG. 2. Box plot of the sum of flinches 0–60 min after the lumbar i.t. administration of either vehicle, 0.3 nmol, 1 nmol, 3.3 nmol or 10 nmol nociceptin, delivered 10 min before the formalin test ($n = 6$ per group). The box represents the interquartile range, the line within the box the median, the dotted line the arithmetic mean. The ends of the 'whiskers' show maximum and minimum. *Statistically significant mean difference ($\alpha = 0.05$).

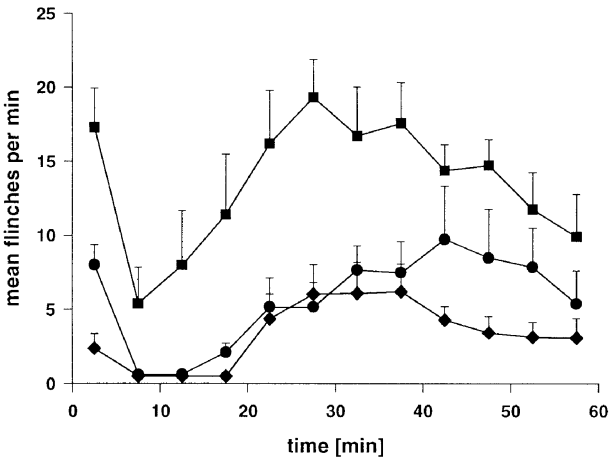

FIG. 3. Time course of flinching behaviour after the administration of either vehicle lumbar i.t. + naloxone 3 mg/kg i.p. (■), 10 nmol nociceptin lumbar i.t. + naloxone 3 mg/kg i.p. (●), or 10 nmol nociceptin + vehicle lumbar i.t. (◆), $n = 6$ per treatment group. Lumbar i.t. administration of nociceptin/vehicle was performed 10 min before i.p. administration of naloxone/vehicle 3–2 min before the formalin test. The data are presented as the mean + s.e.m. For graphical clarification flinches are shown as averages/5 min intervals.

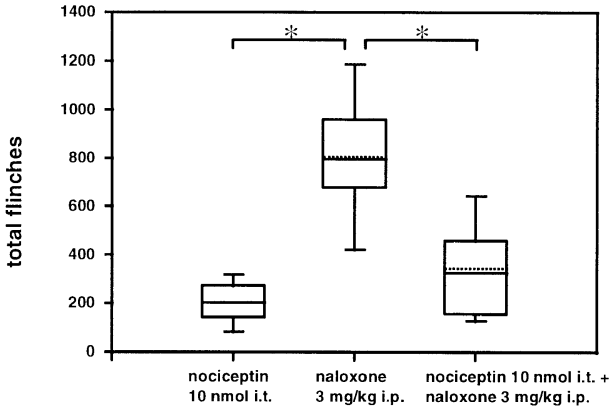

FIG. 4. Box plot of the sum of flinches 0–60 min after the administration of either 10 nmol nociceptin + vehicle lumbar i.t., vehicle lumbar i.t. + naloxone 3 mg/kg i.p., or 10 nmol nociceptin lumbar i.t. + naloxone 3 mg/kg i.p., $n = 6$ per treatment group. The box represents the interquartile range, the line within the box the median, the dotted line the arithmetic mean. The ends of the 'whiskers' show maximum and minimum. *Statistically significant mean difference ($\alpha = 0.05$).

Total flinches were analysed by one-way ANOVA for independent measures provided that the assumptions of homogeneity of variances and normality were not grossly violated. ANOVA was followed by a *post hoc* test (Scheffé). The α level was set at 0.05. Statistical evaluation was performed using SPSS for Windows 7.0.

Results

Effects of 0.3–10 nmol nociceptin on flinching behavior: The recordings of flinches clearly demonstrated a biphasic behaviour in the control group as well as in the nociceptin-treated groups (Fig. 1). The time course following 10 nmol nociceptin showed that flinching during both phases, the short-lasting first and the longer-lasting second phase, was reduced compared with the vehicle-treated group. ANOVA for independent measures of total flinches (sum of all flinches of an individual rat) showed statistically significant differences in treatment means ($F(4,25) = 7.76$, $p < 0.05$). The *post hoc* test identified statistically significant mean differences of the highest nociceptin dose (10 nmol) *vs* vehicle and *vs* 0.3 and 1 nmol nociceptin. Compared with vehicle (mean 939 flinches), animals treated wtih 0.3 nmol nociceptin showed a small increase in total flinches (mean 1002). Following administration of 1, 3.3 or 10 nmol nociceptin mean total flinches decreased dose-dependently by 86, 293 and 597 flinches *vs* vehicle, respectively (group means: dotted lines in the boxes of the box plot, Fig. 2).

Lack of reversal of nociceptin effects by naloxone: The recordings of flinches also demonstrated a clear biphasic behaviour in the naloxone-, nociceptin- and naloxone + nociceptin-treated groups (Fig. 3). The time course following administration of 10 nmol nociceptin and 10 nmol nociceptin + 3 mg/kg naloxone showed that flinching was reduced compared with the group receiving 3 mg/kg naloxone alone. Mean total flinches with naloxone alone were 809, with nociceptin alone 202 and with nociceptin + naloxone 341. ANOVA for independent measures of total flinches showed statistically significant differences in treatment means ($F(2,15) = 14.47$, $p < 0.05$). The *post hoc* test identified statistically significant mean differences between the naloxone- and nociceptin + naloxone-treated groups as well as between the naloxone- and nociceptin-treated groups. No statistically significant mean difference was detected between the nociceptin- and nociceptin + naloxone-treated groups. When flinching during the formalin test was divided into phases (0–10 min, 11–40 min, 41–60 min[10,11]) and these phases were tested separately in an exploratory analysis no statistically significant differences were seen (data not shown).

The flinching-time courses of vehicle administered by the lumbar i.t. route in the first study part (1) and of systemic naloxone in the second study part (2) were similar. The time courses of the effects of nociceptin 10 nmol administered i.t. (1) and 10 nmol nociceptin lit. + systematic naloxone were also similar. No systematic behavioural changes that could have indicated motor impairment during or after the formalin test were observed.

Discussion

This study was performed in order to investigate the effects of spinally delivered nociceptin on flinching

behaviour in the rat formalin test. According to the results in behavioural tests with nociceptin administered i.c.v. in mice, Meunier *et al.*[1] and Reinscheid *et al.*,[2] who described a hyperreactivity response to noxious stimuli rather than antinociception, an increase in flinching behavior as surrogate for increased nociception could have been expected. In accordance with *in vitro* studies which demonstrated inhibitory effects of nociceptin on dorsal raphe nucleus neurones of rats[6] and in a human neuroblastoma cell line[7] we observed a decrease in flinching behaviour with spinally delivered nociceptin in rats. With doses of 1, 3.3 and 10 nmol nociceptin, flinches over the entire observation period decreased dose-dependently. In the formalin test a decrease in flinching behaviour is interpreted as surrogate for antinociception. Such effects occur with e.g. morphine[12] which reduces flinching behaviour in the short-lasting first and the longer-lasting second phase of the formalin test. In contrast to morphine[13] the effects of nociceptin (10 nmol i.t.) were not reversed by a high dose of naloxone (3 mg/kg i.p.).[14] In a recent study by Xu *et al.*,[15] 1 and 10 μg (0.55 and 5.52 nmol) nociceptin i.t. caused strong and sustained depression of a spinal nociceptive flexor reflex in decerebrated rats. These effects were not reversed by naloxone or antagonists of α_2-adrenergic and GABA receptors. Furthermore a potent and prolonged thermal antinociception was observed on the tail flick test (increased latencies) after 1 and 10 μg i.t. nociceptin in rats; neither dose of nociceptin caused any motor impairment.[15] We also observed no systematic behavioural changes that could have been indicative of motor impairment during or after the formalin test. Thus the results of Xu *et al.*,[15] together with our data, provide strong evidence for antinociceptive effects in two different behavioural tests in rats. Possible reasons for the differing results compared from those obtained in studies by other authors[1,2,5] include different sites of administration, different doses and different species used. The discrepancies therefore need further elucidation.

Conclusion

We observed a decrease in flinching behaviour with spinally delivered nociceptin in the rat formalin test. We therefore conclude that nociceptin has antinociceptive effects in this experimental model.

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General Summary
Nociceptin is a putative endogenous agonist of the opioid receptor-like ORL1 receptor but it does not show appreciable binding to opioid receptors. In behavioural tests in mice nociceptin induced rather hyperreactivity to noxious stimuli than antinociceptive effects. The aims of this study were to investigate the effects of spinally delivered nociceptin on the flinching behaviour in the rat formalin test, which is a well documented experimental pain model, and to determine whether these effects were influenced by the concomitant systemic administration of the opioid receptor antagonist naloxone. Following the administration of 1, 3.3 or 10 nmol nociceptin mean total flinches decreased dose-dependently. The effects of 10 nmol nociceptin were not reversed by a high dose of naloxone. There was a decrease in flinching behaviour with spinally delivered nociceptin in the rat formalin test. We therefore conclude that nociceptin has antinociceptive effects in this experimental model.