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Effects of maternal separation on brain nociceptin/orphanin FQ peptide levels in male Wistar rats

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Abstract

Environmental manipulation early in life may induce persistent alterations in adult behaviour and physiology. In this study, we investigated the long-term effects of daily maternal separation, Days 1–21, on brain immunoreactive nociceptin/orphanin FQ (ir-N/OFQ) levels in male Wistar rats. The rat pups were separated in litters for 360 min (MS360) or 15 min (H15). Control rats were left undisturbed until weaning. Peptide levels were measured at 10 weeks of age. In the hypothalamus and periaqueductal gray, MS360 induced an increase in ir-N/OFQ levels in comparison with control rats. H15 rats had increased ir-N/OFQ levels in the hypothalamus and the medial prefrontal cortex compared with control animals. The rats were also tested at two occasions in an elevated plus-maze. An increased anxiety-like behaviour was shown in MS360 rats at weaning, whereas a decreased anxiety response was found at 9 weeks of age compared with control rats. The study shows that early life experiences induce long-term effects on behaviour, as well as brain N/OFQ levels.

Keywords: Maternal separation; Neonatal handling; Stress; Nociceptin/orphanin FQ; Elevated plus-maze

1. Introduction

Chronic exposure to stress can induce long-lasting alterations in the behaviour of humans and experimental animals. An increased vulnerability to stress is discussed as a major contributing factor in human psychiatric disorders, such as anxiety and depression (Arborelius et al., 1999; Heim et al., 1997). One approach to the identification of potential animal models of psychiatric disorders involves manipulation of the environment of the individual during its postnatal development, followed in adulthood by behavioural and neurobiological screening (Ellenbroek and Cools, 1998; Feldon and Weiner, 1991). Brief (3–20 min) daily separation of the pups from the dam until weaning, referred to as neonatal handling (H), results in the attenuation of neuroendocrine responses to stress both at the behavioural and the neurochemical levels (for reviews, see Anisman et al., 1998; Lehmann and Feldon, 2000; Meaney et al., 1996). Rats that have been subjected to early short daily separations show less anxiety-like behaviour (Ader, 1968; Ploj et al., 1999; Vallee et al., 1997; Wakshlak and Weinstock, 1990). Prolonged periods (>1 h) of maternal separation (MS) have been reported to induce opposite effects compared with short periods of separation, although contrary findings have been reported. In terms of behavioural effects, increased, decreased or no effect on emotionality and anxiety have been reported (for a review, see Lehmann and Feldon, 2000). Possible explanations for these contradictory results may be the different experimental conditions used, such as the duration and frequency of separation, age and different models used to evaluate the effects of separation.

We have previously reported that a daily 15-min separation during Postnatal Days (PNDs) 1–21 induces long-term alterations in brain immunoreactive (ir) opioid peptide levels in male Sprague–Dawley rats (Ploj et al., 1999), as well as opioid and nociceptin/orphanin FQ (N/OFQ) levels in female Sprague–Dawley rats (Ploj et al., 2001). The recently discovered neuropeptide N/OFQ (Meunier et al., 1995; Reinscheid et al., 1995) has structural similarities with endogenous opioid peptides but has different physiological effects. The wide distribution of N/OFQ and its receptor (ORL1) in the central nervous system suggests that it may

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be involved in the control of several biological functions (Lachowicz et al., 1995; Schulz et al., 1996). So far, N/OFQ has been shown to induce a variety of behavioural effects, including interference with motor performance, stimulation of feeding, hyperalgesia, reversal of stress-induced analgesia and analgesia (Walker and Koob, 1997). N/OFQ has also been implicated in stress-regulatory functions and may have a potential role in modulating anxiety and/or behavioural responses to various stressors. For example, central administration of N/OFQ decreases anxiety-related behaviour in a variety of tests in rats and mice (Ciccocioppo et al., 2001; Griebel et al., 1999; Jenck et al., 1997). In addition, N/OFQ gene deficient mice consistently show impaired adaptation to repeated stress (Köster et al., 1999).

The possible involvement of N/OFQ in stress responses raised the question whether this peptide system could be affected by early life experiences such as MS and H. The aim of this study was to investigate whether a daily short (15 min) and long (360 min) separation, H15 and MS360, respectively, during PNDs 1–21 could affect the N/OFQ peptide system in various brain regions, directly or indirectly implicated in emotional processing in male Wistar rats. N/OFQ was measured, using radioimmunoassay, in brain tissue extracts 7 weeks after the respective separation procedure. In order to investigate anxiety-like behaviour, the rats were also tested at two occasions in an elevated plus-maze.

2. Materials and methods

2.1. Animals

Time-mated pregnant Wistar rats were obtained from B&K Universal, Sweden for arrival at our animal department on Gestational Day 15. Upon arrival, the dams were singly housed in standard macrolon cages ($59 \times 38 \times 20$ cm) containing nesting material and maintained on standard pellet food and water ad libitum. All animals were housed in a temperature (22 ± 2 °C)- and humidity ($50 \pm 5\%$)-controlled animal room, only used for this experiment, on a 12:12-h light–dark cycle (light on at 06:00 h). All animal experiments were treated under an approved protocol in accordance with the Swedish Animal Protection Legislation.

2.2. Neonatal handling, 15 min and maternal separation, 360 min

Wistar rats weighing 266–354 g at the end of the experiment were used. The litters were sexed and culled (nine males and two females per litter) on Day 0 (day of birth = Day 0). A total number of 236 rats were used. Some of these rats were used to examine effects on transmitter systems other than N/OFQ. The rats were randomly assigned to either one of two treatments: 360 min separation (MS360), 15 min handling (H15) or no treatment (control rats). Seven litters were used for H15 treatment, six litters for MS360 and nine litters were controls. Three of the control litters were assigned to establish eye opening and for weight measurements. The remaining control rats were not disturbed until weaning, except for cage changes with clean bedding material once a week. In all tests, animals were taken from several litters to avoid the use of littermates in the same treatment group. The separation occurred once a day for the first three postnatal weeks. First, the dam and then the pups were removed from the nest. Each litter was placed in macrolon cages ($26 \times 20 \times 14$ cm) containing wood chip bedding material and moved to an adjacent room (25 °C). The cages, in which the pups were separated, were changed every day during the experiment. After either 15 or 360 min, first the pups and then the dam were returned to their home cage. During the separation procedure, the dams of rat pups separated for 360 min were returned to their home cages. Separation sessions were always performed in the same room by the same experimentors (two persons), who were the only persons with permission to enter the animal rooms during the experiment. The separation treatment occurred between 09:00 and 15:00 h daily, and the rat pups ($n = 169$) were inspected every day to establish the day for eye opening. On PND 22, the animals were weighed and weaned. In order to measure anxiety-like behaviour, the rats were tested in an elevated plus-maze. The rats were thereafter housed in same-treatment groups of three to five males per cage. On PND 61 or 62, the rats were tested in a second trial in the elevated plus-maze test. Only male rats were used for the behavioural and neurochemical analysis. At 7 weeks after the separation procedure and 1 week after the last plus-maze trial, the rats were sacrificed by decapitation, and the brains were taken out and dissected.

2.3. Dissection

Rats from different litters from the H15 ($n = 10$), MS360 ($n = 10$) and the control ($n = 10$) groups were used to evaluate the effects of neonatal manipulation on brain N/OFQ levels. The hypothalamus was removed from the brain using a small forceps. The brain was then sliced manually, and the following regions were dissected out with the guidance of a brain atlas: frontal cortex, medial prefrontal cortex, nucleus accumbens, striatum, hippocampus, amygdala, substantia nigra, ventral tegmental area (VTA) and the periaqueductal gray (PAG). The tissues were immediately frozen on dry ice and stored at −80 °C until the time for peptide analysis.

2.4. Extraction and purification of tissue extracts

Tissue extraction was performed with 1 M acetic acid. The samples were heated at 95 °C for 5 min and, after cooling on ice, homogenised with sonication. The samples were reheated at 95 °C for 5 min, cooled on ice and then centrifuged for 15 min at $12\,000 \times g$. The supernatants were applied onto 1-ml ion exchange columns containing SP

Sephadex C-25 gel (Amersham Pharmacia Biotech, Uppsala, Sweden), and N/OFQ was eluted with a buffer containing 1.6 M pyridine and 1.6 M formic acid. The fractions were dried in a vacuum centrifuge before peptide analysis.

2.5. Radioimmunoassay

ir-N/OFQ tissue levels were measured with a specific radioimmunoassay, using a procedure previously described in detail (Ploj et al., 2000). The samples were dissolved in methanol/0.1 M HCl (1:1), then the sample (25 μl), antiserum (100 μl) and ^{125}I-labelled Tyr^{14}-N/OFQ (100 μl) were incubated for 24 h at 4 °C. The antiserum and labelled peptide were diluted in a gelatin buffer. The tracer peptide was labelled with ^{125}I using chloramine T and purified with reversed-phase HPLC. To separate free and antibody-bound peptides, 100 μl of a sheep-antirabbit antiserum (Pharmacia Decanting Suspension 3; Pharmacia Diagnostics, Uppsala, Sweden) was added, and the samples were incubated for 1 h at 4 °C. After centrifugation for 10 min at 12 000×*g*, the radioactivity of the pellet was measured in a gamma counter. The antiserum 96:2+ was used in a final dilution of 1:112,500. Crossreactivity with N/OFQ (1–13) was 0.5%, with nocistatin and the opioid peptides, dynorphin (DYN) A(1–17), DYNB, DYNA(1–6), DYN32, DYNB29, Met-enkephalin, Met-enkephalinArg^{6}Phe^7, Leu-enkephalin and β-endorphin less than 0.1%.

2.6. Elevated plus-maze

Two to three male pups from different litters from the H15 (n=12–13), MS360 (n=12–14) and the control groups (n=12–13) were tested. The elevated plus-maze consisted of four arms, each 40 cm long and 10 cm wide, arranged in the shape of a plus sign, elevated 52 cm off the ground. Two opposite arms were open, whereas the other two were closed, with 43-cm-high walls. The entire plus-maze was made of metal with black rubber walls and floor. To begin the test, each rat was placed in the centre of the maze, facing an open arm, whereafter the experimentor left the room. The area inside the centre portion (10 × 10 cm) was not considered as either an opened or closed arm. To consider an animal being located within either the open, closed or centre sections of the maze, all four of the animal's paws had to be within one of these defined sections. After the test of each rat, the maze was cleaned with water. Each rat was tested for 5 min at PND 22 and PND 61 or 62, respectively. At PND 61 or 62, the rats were isolated in a smaller cage for 30 min before the start of the test to enhance the stress response. The behaviour on the maze was recorded via a Panasonic Super Dynamic WV-BP 550/B video camera mounted in the ceiling above the centre of the maze and relayed to a SABA M3705M monitor and a Panasonic A6-TL300 VCR. The room temperature was 22 ± 2 °C, and dimmed light was used during the test. The following data were recorded: number of total and open-arm entries, open-arm entries expressed as the percentage of total arm entries, total time spent in the arms, time spent in open arms, time spent in open arms expressed as the percentage of total time in the arms and the latency to the first open-arm entry. As a further measurement of stress response, faecal boli were counted at the end of each trial.

2.7. Statistical analysis

Data are expressed as means±S.E.M. For comparisons of the day for eye opening, number of faecal boli and ir-N/OFQ levels, ANOVA Factorial, combined with the Fischer's post hoc test, was used. The nonparametric Mann–Whitney U test was used to analyse the behavioural data from the elevated plus-maze test. Differences were considered statistically significant at $P \leq .05$. The StatView 4.5 computer software was used for all statistical analyses.

3. Results

The body weight increase during the experiment was similar in all rat groups, and no differences in body weight among the three groups were found at PND 22 or at 10 weeks of age.

The time point for eye opening is shown in Fig. 1. Most rat pups opened their eyes on PNDs 14–15; however, 20% of MS360 pups opened their eyes on PNDs 16–17 compared with H15 and control rats (7% and 3%, respectively). ANOVA showed significant group differences regarding the day for eye opening [$F(2,166) = 5.50$, $P < .01$]. Post hoc analysis revealed that MS360 opened their eyes later compared with both H15 ($P < .01$) and control rats ($P < .01$). No

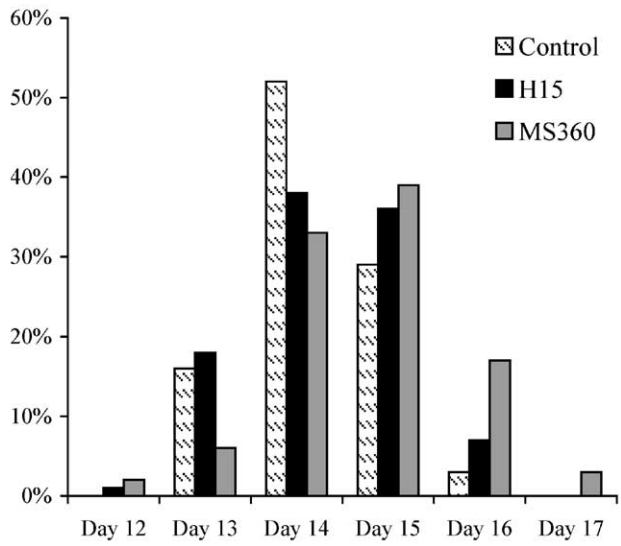

Fig. 1. Time point for eye opening in the MS360, H15 and control groups. The figure illustrates the percentage of animals in each group opening their eyes during PNDs 12–17.

significant difference in the day for eye opening was seen between H15 and control rats.

The behavioural data from the plus-maze are illustrated in Figs. 2A–B and 3, and all data are summarised in Table 1. The plus-maze test at PND 22 showed that MS360 animals made fewer entries into the open arms and waited longer to first enter the open arms than the control animals. No differences were observed between the control and H15 animals or the H15 and MS360 animals. No statistically significant differences were seen in the total number of arm entries (activity), total time spent on all arms or the time spent on open arms among the groups. The plus-maze test at PND 61 or 62 showed that the MS360 animals spent more time in the open arms compared with control animals. No differences were observed between the control and H15 animals or the H15 and MS360 animals. No differences among the groups were seen in the number of total and open-arm entries, total time in all arms or latency to first enter the open arms. The number of faecal boli was approximately the same in the three groups at PND 22 [$F(2,32)=0.58$, $P=.56$] and PND 61 or 62 [$F(2,35)=0.65$, $P=.53$].

The ir-N/OFQ levels in various brain areas of the MS360, H15 and the control rats are shown in Table 2.

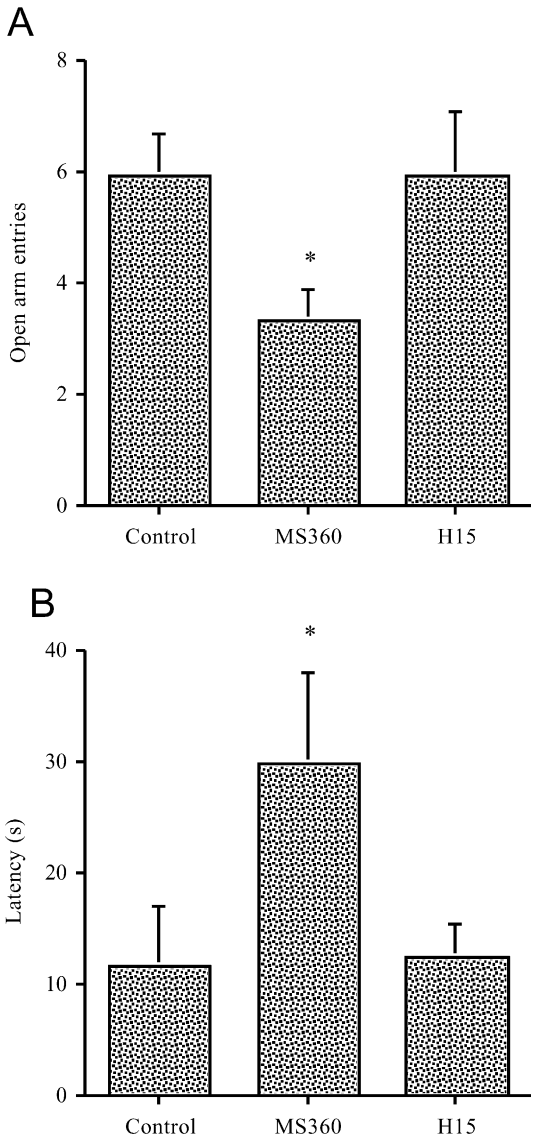

Fig. 2. (A and B) Number of entries into the open arms (means ± S.E.M.) and latency (s) to the first open-arm entry (means ± S.E.M.) in the elevated plus-maze test at PND 22 ($n=12$ in each group). *$P<.05$ compared to control animals (Mann–Whitney U test).

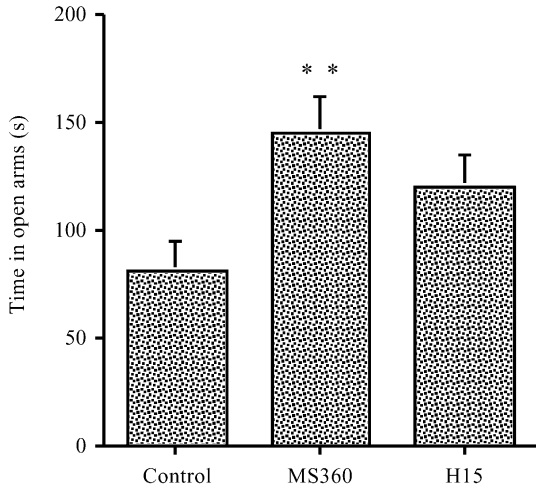

Fig. 3. Time (s) spent in open arms (means ± S.E.M.) in the elevated plus-maze test at PND 61 or 62 ($n=13–14$ in each group). **$P<.01$ compared to control animals (Mann–Whitney U test).

Table 1
Effects of MS360 and H15 on the elevated plus-maze behaviour at PND 22 and PND 61 or 62

	MS360	H15	Control
Test at PND 22			
No. of arm entries			
Total	9.7 ± 1.1	12.3 ± 1.6	11.9 ± 0.8
Open arms	3.3 ± 0.6*	5.9 ± 1.2	5.9 ± 0.8
Open arms (% of total)	33.9 ± 4.7*	43.0 ± 5.3	49.1 ± 4.9
Time spent in arms (s)			
Total	234.2 ± 11.3	244.0 ± 9.5	244.6 ± 7.2
Open arms	57.3 ± 13.9	73.4 ± 10.8	93.6 ± 15.2
Open arms (% of total)	23.8 ± 5.7	31.0 ± 4.7	39.2 ± 6.6
Latency to enter open arms (s)	29.7 ± 8.4*	12.2 ± 3.2	11.4 ± 5.6
Test at PND 61 or 62			
No. of arm entries			
Total	10.1 ± 0.8	11.0 ± 1.0	10.4 ± 0.9
Open arms	5.4 ± 0.7	5.2 ± 0.8	4.3 ± 0.9
Open arms (% of total)	52.9 ± 5.2	45.1 ± 5.5	38.7 ± 5.7
Time spent in arms (s)			
Total	267.2 ± 3.4	261.4 ± 4.6	259.3 ± 6.0
Open arms	144.7 ± 17.8**	119.0 ± 16.7	80.6 ± 14.4
Open arms (% of total)	54.3 ± 6.6*	46.0 ± 6.5	31.4 ± 5.7
Latency to enter open arms (s)	25.0 ± 11.6	21.1 ± 5.3	20.4 ± 9.6

Data are expressed as group means ± S.E.M. ($n=12–14$).
* $P<.05$, compared with control animals (Mann–Whitney U test).
** $P<.01$, compared with control animals (Mann–Whitney U test).

Table 2
ir-N/OFQ levels in various brain areas in rats 7 weeks after the MS360 and H15 procedure

Region	MS360	H15	Control
Hypothalamus	19.43 ± 0.91 (10)**	20.97 ± 1.16 (10)***	15.05 ± 0.90 (10)
Frontal cortex	3.05 ± 0.24 (8)	2.89 ± 0.23 (8)	2.63 ± 0.38 (9)
Medial prefrontal cortex	2.42 ± 0.27 (9)	3.04 ± 0.22 (10)**	2.08 ± 0.18 (9)
Nucleus accumbens	4.33 ± 0.41 (8)	3.61 ± 0.37 (7)	3.21 ± 0.27 (9)
Striatum	2.14 ± 0.16 (10)	2.07 ± 0.13 (10)	1.87 ± 0.05 (9)
Amygdala	7.97 ± 1.80 (10)	4.27 ± 0.51 (7)	6.13 ± 0.95 (7)
Hippocampus	5.40 ± 0.13 (10)	5.72 ± 0.15 (10)	5.34 ± 0.17 (8)
Substantia nigra	3.30 ± 0.24 (9)	3.39 ± 0.36 (8)	3.88 ± 0.34 (9)
VTA	3.43 ± 0.43 (7)	3.02 ± 0.31 (9)	3.42 ± 0.50 (8)
PAG	15.53 ± 1.65 (8)*	11.52 ± 1.31 (8)	10.24 ± 1.44 (7)

Values represent the means ± S.E.M. (n) and are expressed as fmol/mg tissue.
* $P < .05$, compared with control animals (ANOVA, Fisher's post hoc test).
** $P < .01$, compared with control animals (ANOVA, Fisher's post hoc test).
*** $P < .001$, compared with control animals (ANOVA, Fisher's post hoc test).

ANOVA indicated significant group effects in the hypothalamus [$F(2,27) = 9.45$, $P < .001$], medial prefrontal cortex [$F(2,25) = 4.65$, $P < .05$] and the PAG [$F(2,20) = 3.48$, $P = .05$]. Post hoc analysis revealed that rats in the MS360 group had higher ir levels of N/OFQ in the hypothalamus and the PAG compared with those of the control rats. H15 rats had increased ir-N/OFQ levels in the hypothalamus and the medial prefrontal cortex compared with control rats. No differences among the groups were seen in the frontal cortex, nucleus accumbens, striatum, hippocampus, amygdala, substantia nigra or the VTA.

4. Discussion

Effects of the neonatal manipulations MS and H on the N/OFQ peptide system in the male rat brain have, to our knowledge, not previously been reported. We have earlier described long-term changes in PAG ir-N/OFQ peptide levels in female Sprague–Dawley rats after H (Ploj et al., 2001). This study on male rats presents further evidence that H15 and, in addition, MS360 can induce long-lasting changes in the N/OFQ peptide system in the rat brain.

The current protocols for H15 and MS360 were found to cause a delay in time for eye opening in the MS360 rats compared with both H15 rats and control rats, suggesting an effect of MS360 on the normal development in the pup. Feeding behaviour was seemingly normal, and no body weight differences were detected among the three groups during the experiment.

Previous reports on long-term behavioural effects of MS, using emotional and anxiety tests, have been contradictory. For example, 360 min of repeated MS has been reported to decrease anxiety in the open-field behaviour test but not in the plus-maze test (Kaneko et al., 1994), while 180 min of repeated MS was found to increase anxiety-like behaviour in the plus-maze (Wigger and Neumann, 1999). However, several others failed to find an effect of repeated MS on emotional behaviour (Biagini et al., 1998; Crnic et al., 1981; Ogawa et al., 1994; von Hoersten et al., 1993). To evaluate short- and long-term effects on anxiety-like behaviour in the present study, the animals were tested in an elevated plus-maze at two occasions during the study. In the first trial at PND 22, MS360 rats displayed a higher degree of anxiety-like behaviour than the control animals in terms of number of entries and the latency to first enter the open arms. In order to investigate long-term behavioural effects of MS, the rats were tested in an additional plus-maze trial at PND 61 or 62, 1 week before decapitation and subsequent neurochemical analysis. It was hypothesised that the higher degree of anxiety-like behaviour shown at PND 22 would persist in the MS360 animals. In order to enhance the anxiety response and generate a more pronounced effect in the plus-maze test, a short isolation in a separate cage was performed on all rats prior to testing at PND 61 or 62. Unexpectedly, MS360 animals now showed less anxiety-like behaviour and spent more time in the open arms than control rats. Although not statistically significant, H15 rats displayed similar results and in the same direction as MS360. Thus, although presenting a more pronounced anxiety-like behaviour at PND 22, the MS360 rats seemed to respond with less anxiety to the short isolation and in the plus-maze test at PND 61or 62. At this second test, the control rats instead are those showing the highest degree of anxiety. The most probable explanation for the behaviour of the control rats is that these rats, as opposed to the H15 and MS360 rats, have never been handled other than during cage changes in this study. The control rats may therefore be expected to show a higher anxiety-like behaviour in response to experimentor handling.

Interestingly, when comparing our results from the neurochemical analysis with the behavioural data, it was found that rat pups exposed to MS360 have increased ir-N/OFQ levels in the hypothalamus, compared with control rats. Surprisingly, the ir-N/OFQ levels were also elevated in the H15 rats compared with control rats. Accordingly, both MS360 and H15 rats, those that showed least anxiety-like behaviour in the plus-maze test, had high ir-N/OFQ levels in

the hypothalamus compared with control rats. Again, the nonhandled control rats differed and expressed lower ir-N/OFQ levels than rats in the other groups. ORL1 receptors are present in many nuclei of the hypothalamus and may mediate effects on hormonal regulation and modulation of the hypothalamic–pituitary–adrenal (HPA) axis (Ciccocioppo et al., 2001; Devine et al., 2001; Lachowicz et al., 1995; Mollereau et al., 1994). The HPA axis is clearly affected by H, as evidenced by a reduced responsivity to stressors, whereas MS induces the opposite effect, although both hyper- and hyporesponsiveness have been reported after MS (for a review, see Lehmann and Feldon, 2000). The high ir-N/OFQ levels in the hypothalamus, found in the present study, may be related to these consequences of neonatal manipulation.

Increased ir-N/OFQ levels in H15 rats were also seen in the medial prefrontal cortex compared with controls. The medial prefrontal cortex has been shown to modulate emotional behaviour and stress responses, suggesting that dysfunction in this area may be involved in the pathogenesis of depressive/anxiety symptoms (for a review, see Drevets, 2000). The higher ir-N/OFQ levels observed in H15 rats may indicate an involvement of N/OFQ in medial prefrontal cortex emotional processing. The midbrain PAG has been identified as a region containing distinct neural networks, which initiate emotional coping strategies. A dense plexus of N/OFQ nerve fibres and terminals can be found within the PAG in mice and rats (Schulz et al., 1996), suggesting that N/OFQ might participate in PAG-mediated modulation of pain and stressful stimuli. Higher ir-N/OFQ and opioid peptide levels have been reported in the PAG in female Sprague–Dawley rats after H15 (Ploj et al., 2001). In the present study, using male Wistar rats, ir-N/OFQ levels were unaffected by H15. These results indicate gender and/or strain differences in the response to neonatal manipulation, as also shown earlier (Ploj et al., 2001). MS360 rats had increased ir-N/OFQ levels in the PAG compared with control rats and, as noted, the MS360 rats also exhibited less anxiety-like behaviour in the plus-maze trial prior to neurochemical analysis.

In conclusion, this study shows that daily disturbance of the mother–infant relationship during the first three neonatal weeks causes long-term changes in the brain N/OFQ peptide system. The present findings, showing effects on ir-N/OFQ levels in certain brain areas after neonatal manipulation, and the previously suggested stress regulatory functions of N/OFQ, may point to a potential role of the N/OFQ system in emotional stress responses and in the consequences of MS and/or H.

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