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Effects of neonatal handling on nociceptin/orphanin FQ and opioid peptide levels in female rats

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Abstract

Animals exposed to short periods of handling during the critical period of development, i.e., the first 21 days of life in rats, show attenuated neuroendocrine responses to stress in adult life. We have previously reported long-term changes in brain dynorphin (DYN) peptide levels in male Sprague–Dawley rats after neonatal handling. The purpose of this study was to investigate whether neonatal handling, 15-min individual separation from the mother during postnatal days 1–21, can induce long-term changes in DYNB, Met-enkephalin Arg^6Phe^7 (MEAP) and nociceptin/orphanin FQ (N/OFQ) immunoreactive (ir) levels in female Sprague–Dawley rats. The peptides were measured in brain and pituitary gland 2 months after the handling procedure. The results reveal that handled (H) rats had increased ir levels of N/OFQ, DYNB and MEAP in the periaqueductal gray (PAG) as compared to nonhandled (NH) controls. Furthermore, H rats had decreased ir levels of DYNB in the frontal cortex and in the amygdala. In contrast to previous findings in male rats, DYNB levels were unaffected in areas related to the hypothalamo–pituitary–adrenal (HPA)-axis. The results indicate that a manipulation early in life can induce persistent neurochemical changes in the N/OFQ and opioid peptide system in female Sprague–Dawley rats.

Keywords: Neonatal handling; Stress; Opioids; Nociceptin/orphanin FQ

1. Introduction

It is well known that various stressful stimuli cause a powerful behavioural and neurochemical response. Chronic exposure to stress can induce long-lasting alterations in the behaviour of humans and experimental animals. Severe forms of stress early in life can be detrimental and even cause mental illness (Arborelius et al., 1999), whereas milder forms of stress such as gentle handling induce positive behavioural effects (Anisman et al., 1998; Hall, 1998; Ladd et al., 2000). The neurochemical mechanisms underlying these long-lasting changes in behaviour are not completely understood. Studies on stress-induced effects on neuropeptides mostly describe short-term effects of acute stress, whereas knowledge about long-term effects of stress exposure is poor.

The endogenous opioid peptides act at the μ, δ and κ receptors to mediate a variety of physiological effects, such as pain modulation (Terenius, 1992), motivation, reward (Nylander and Silberring, 1998) and neuroendocrine secretion (Pfeiffer and Herz, 1984) in the central and peripheral nervous system. Several studies have shown that stressors modify the endogenous opioid system. Different kinds of stressors, such as social isolation (Vanderschuren et al., 1995), electric shock (Watkins et al., 1992), forced swimming (Vanderah et al., 1992), restraint (Calcagnetti and Holtzman, 1992) and forced social interactions (Dijkstra et al., 1992), have been shown to activate the endogenous opioid system, which in turn induces responses at the neuroendocrine and behavioural level.

The recently identified nociceptin (Meunier et al., 1995) or orphanin FQ (N/OFQ) peptide (Reinscheid et al., 1995) has a structure similar to that of the opioid peptides, but it does not bind to opioid receptors, and opioid peptides do not bind to the N/OFQ receptor, ORL1. N/OFQ, its precursor peptide, and the ORL1 receptor are located in corticolimbic regions involved in the integration of the emotional components of fear and stress, with a pattern distinct from that of opioid peptides and receptors in rodents (Houtani et al., 1996; Lachowicz et al., 1995; Schulz et al.,

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1996). N/OFQ has been shown to induce a wide range of physiological and behavioural effects (Meunier, 1997). For example, endogenous N/OFQ has been reported to be released following acute stress (Devine et al., 1998) and to act as a regulator of acute anxiety responses (Griebel et al., 1999; Jenck et al., 1997).

In the neonatal handling procedure, the rats are handled (H) a short period daily during the first 21 days of life. During this critical period of development handling results in attenuation of neuroendocrine responses to stress both at the behavioural and the neurochemical level, that has consequences for the animals' behaviour later in life. H rats have higher tolerance to stress and show more rapid regulation of physiological stress reactions in comparison with nonhandled (NH) rats (Meaney et al., 1996). In addition to the neurochemical changes caused by handling, behavioural alterations have also been demonstrated. Handling results in decreased emotionality and anxiety in comparison with NH rats (Ader, 1968; Costela et al., 1995; Fernandez-Teruel et al., 1997; Nunez et al., 1995; Ploj et al., 1999; Wakshlak and Weinstock, 1990). Since the handling procedure represents a well-documented method to induce behavioural effects in the animals, effects that persist throughout life (Meaney et al., 1988), it is here used as an experimental model to study whether handling early in life can induce long-term changes in neuropeptide systems. In a previous study, we have reported differences in immunoreactive (ir) dynorphin (DYN) levels between H and NH male Sprague–Dawley rats in the pituitary gland and the brain (Ploj et al., 1999). Here, we extend the studies of handling-induced effects by examining two opioid peptides and the structurally related N/OFQ in various structures, which are implicated directly or indirectly in emotional processing in the brain, 2 months after handling in female Sprague–Dawley rats. The opioid peptides DYNB, derived from proDYN, Met-enkephalin Arg^6Phe^7 (MEAP), a marker of proenkephalin, and the structurally related peptide N/OFQ were measured using specific radioimmunoassays.

2. Materials and methods

2.1. Animals

Pregnant Sprague–Dawley rats (B&K Universal, Sweden) were housed singly in standard macrolon cages and were maintained on food and water ad libitum. The room temperature was controlled at 22 ± 2°C and a 12:12 light–dark cycle (light on 06:00 h) was used. The animals were randomly divided into two experimental groups to which their offspring would be assigned: NH animals, which were not disturbed until weaning, and animals subjected to handling treatment. All animal experiments were performed under an approved protocol in accordance with the Swedish Animal Protection Legislation.

2.2. Neonatal handling

The handling treatment was carried out between postnatal days 1 and 21. The mother and then the pups were removed from the nest. The pups were then put in individual plastic boxes lined with paper towels at room temperature. After 15 min, the pups and then the mother were returned to their home cage. The handling treatment occurred between 09:00 and 12:00 h daily. All pups were weaned and separated by sex on postnatal day 22 and housed in same-treatment groups of five. In this experiment, 10 female H and 10 female NH rats were used. Two months after the handling procedure, all animals were sacrificed by decapitation. The anterior and neurointermediate lobe of the pituitary were taken out and the hypothalamus was removed from the brain, whereafter, the brain was sliced manually and the following structures were dissected out: frontal cortex, nucleus accumbens, hippocampus, striatum, amygdala, substantia nigra, ventral tegmental area (VTA) and the periaqueductal gray (PAG). The tissues were frozen on dry ice and stored at -80°C until the time for peptide analysis.

2.3. Tissue homogenisation and peptide extraction

The tissues were boiled in 1 M acetic acid for 5 min at 95°C, cooled on ice and homogenised by sonication using a Branson Sonifier and then reheated for 5 min at 95°C. The homogenates were cooled on ice and centrifuged for 10 min at $12{,}000 \times g$ in a Beckman GS-15R Centrifuge. The supernatants were saved for chromatography.

2.4. Cation exchange chromatography

The tissue extracts were purified using a cation exchange procedure. The method is routinely used and has been described in detail elsewhere (Christensson-Nylander et al., 1985). The tissue extracts were added onto small cation exchange columns containing SP Sephadex C-25 gel (Pharmacia Diagnostics, Uppsala, Sweden). The peptides were eluted in separate fractions by stepwise elution using a series of buffers containing a mixture of pyridine and formic acid with increasing ionic strength. MEAP elutes in 0.35 M pyridine:0.35 M formic acid, whereas N/OFQ and DYNB elute in 1.6 M pyridine:1.6 M formic acid. The fractions were taken to dryness in a vacuum centrifuge and stored at -80°C.

2.5. Peptide assays

The peptides were analysed using specific radioimmunoassays. The samples and standard DYNB peptide were dissolved in methanol: 0.1 M hydrochloric acid (1:1).

Table 1
Ir peptide levels in different brain regions of rats 2 months after the neonatal handling procedure

	N/OFQ		DYNB		MEAP	
	H	NH	H	NH	H	NH
Anterior lobe	–[a]	–[a]	3.42 ± 0.41 (8)	2.60 ± 0.30 (7)	0.58 ± 0.07 (8)	0.69 ± 0.08 (9)
Neurointermediate lobe	–[a]	–[a]	463.54 ± 58.33 (8)	487.77 ± 65.60 (8)	–[a]	–[a]
Hypothalamus	11.64 ± 0.68 (10)	10.40 ± 0.56 (10)	5.92 ± 0.58 (10)	6.46 ± 0.40 (10)	13.46 ± 2.07 (10)	14.48 ± 1.39 (10)
Nucleus accumbens	2.64 ± 0.32 (9)	2.78 ± 0.35 (8)	15.26 ± 1.07 (8)	14.66 ± 2.24 (8)	42.48 ± 5.48 (10)	52.89 ± 16.36 (8)
VTA	3.75 ± 0.73 (8)	2.85 ± 0.25 (7)	1.36 ± 0.21 (8)	1.29 ± 0.21 (8)	5.91 ± 0.70 (8)	4.45 ± 1.21 (7)
Striatum	1.15 ± 0.15 (8)	0.86 ± 0.07 (10)	3.91 ± 0.45 (8)	5.26 ± 0.49 (10)	11.79 ± 1.56 (8)	19.30 ± 3.63 (8)
Substantia nigra	2.08 ± 0.73 (8)	2.56 ± 0.39 (9)	31.21 ± 3.03 (8)	34.28 ± 2.19 (8)	3.49 ± 0.42 (10)	4.00 ± 0.69 (8)
Frontal cortex	1.14 ± 0.20 (8)	1.49 ± 0.21 (8)	0.40 ± 0.04 ** (8)	0.72 ± 0.09 (7)	1.24 ± 0.12 (8)	1.88 ± 0.35 (10)
Hippocampus	2.30 ± 0.21 (10)	2.14 ± 0.39 (9)	5.06 ± 0.77 (10)	8.37 ± 1.42 (10)	2.85 ± 0.52 (8)	2.73 ± 0.24 (8)
Amygdala	2.25 ± 0.20 (5)	1.91 ± 0.19 (7)	1.86 ± 0.15 * (6)	4.04 ± 0.70 (7)	10.68 ± 1.90 (5)	8.06 ± 2.39 (7)
PAG	5.30 ± 0.45 * (10)	3.91 ± 0.45 (10)	2.30 ± 0.28 ** (10)	1.20 ± 0.12 (10)	18.05 ± 3.53 ** (10)	5.65 ± 0.74 (10)

Values (fmol/mg tissue) represent mean ± S.E.M. (*n*).
[a] The values are below detection limit in the radioimmunoassay.
* $P < .05$ (Student's *t* test).
** $P < .01$ (Student's *t* test).

Twenty-five microliters of sample or standard peptide solution were incubated with 100 μl of antiserum (113+; final dilution 1:562,500) and 100 μl of ^{125}I-labelled DYNB for 24 h at 4°C. The DYNB antiserum did not show cross-reactivity with either DYNA (1–17) or DYNA (1–8). Cross-reactivity with DYNB 29 was 1% and with big DYN 32 100%. Other opioid peptides did not cross-react with the DYNB antiserum. To separate free and antibody-bound peptides, 100 μl of a sheep-antirabbit antiserum (Pharmacia Decanting Suspension, Pharmacia Diagnostics) were added to the samples and, after 1 h incubation at 4°C, the samples were centrifuged for 10 min at 12,000 × *g* in a Beckman GS-15R Centrifuge. The supernatant was discarded and the radioactivity in the remaining pellet was counted in a Wallac 1470 Wizard γ-counter. The tracer peptide was labelled using chloramin T and purified using a high-performance liquid chromatography (HPLC) procedure. The antiserum and labelled peptide were diluted in a gelatin buffer containing 0.15 M NaCl, 0.02% sodium azide, 0.1% gelatine, 0.1% Triton X-100 and 0.1% bovine serum albumin in a 0.05 M sodium phosphate buffer.

The same assay procedure described above for DYNB was used to measure N/OFQ. The N/OFQ antiserum (96:2+) was used in a final dilution of 1:112,500. ^{125}I-labelled Tyr^{14}-N/OFQ was used as the tracer peptide. Cross-reactivity with N/OFQ (1–13) was 0.5%. With nocistatin and the opioid peptides (DYNA (1–17), DYNB, DYNA (1–6), DYN 32, DYNB 29, Met-enkephalin, Leu-enkephalin, MEAP and β-endorphin), it was less than 0.1%.

Samples subjected to MEAP assay were oxidised prior to the radioimmunoassay procedure. Samples were dissolved in 100 μl 1 M acetic acid, to which 10 μl of 30% H_2O_2 were added. They were then incubated at 37°C for 30 min and dried in a vacuum centrifuge. Twenty-five microliters of sample or standard peptide solution, 100 μl of antiserum and 100 μl of ^{125}I-labelled MEAP were incubated for 24 h at 4°C. The MEAP antiserum (90:3D II) was diluted 1:180,000. Cross-reactivity with Met-enkephalin, Met-enkephalin-Arg^6, Met-enkephalin-Arg^{6}Gly^{7}Leu^8, Leu-enkephalin and DYN A (1–6) was < 0.1%. The antiserum and labelled peptide were diluted in a gelatin buffer containing 0.15 M NaCl, 0.025 M EDTA, 0.1% gelatine and 0.1% bovine serum albumin in 0.05 M sodium phosphate buffer. A charcoal suspension (200 μl), consisting of 250 mg charcoal and 25 mg dextran T-70 in 100 ml of 0.05 M sodium phosphate buffer, was added to the samples, which were incubated for 10 min and then centrifuged for 2 min at 12,000 × *g* in a Beckman GS-15R Centrifuge. The radioactivity in a 300 μl aliquot of the supernatant was measured in the γ-counter.

2.6. Statistical analysis

Comparisons of the means in the H rat group and the NH group were analysed with Student's unpaired *t* test using the Statview 4.5 computer program. The level of statistical significance was set at $P < .05$.

3. Results

The ir N/OFQ, ir DYNB and ir MEAP peptide levels in the pituitary gland and in various brain areas of H and NH rats are shown in Table 1. The tissue peptide levels were measured 2 months after the handling period. Reduced ir DYNB levels were detected in the frontal cortex (44% reduction, $P < .01$) and in the amygdala (54% reduction, $P < .05$) in H rats compared with NH rats. Ir DYNB levels were also affected in the PAG, where higher (92% increase, $P < .01$) levels were found in the H rats. In addition to DYNB, ir MEAP levels (219% increase, $P < .01$) and ir N/OFQ levels (35% increase, $P < .05$) were altered in the PAG in H rats compared with NH rats. The ratio of DYNB and MEAP was calculated for all structures

to examine possible divergent effects of handling on these opioid peptides. In the amygdala, where the ir DYNB levels were reduced, ir MEAP levels were affected in the opposite direction (33% higher levels). The DYNB/MEAP ratio was also shown to be significantly lower ($P < .01$) in H rats (0.18 ± 0.03 as compared to 1.13 ± 0.21 in the control rats). In the other examined areas, the DYNB/MEAP ratio was unchanged. In some brain areas, quite large differences were observed between H and NH control rats. However, due to fairly large variation within groups, these differences did not reach statistical significance and are therefore mentioned below, but not further discussed in any detail. In the VTA, both ir N/OFQ and MEAP levels were higher (32% and 33%, respectively) in H rats than in NH rats. The ir levels of opioid peptides were lower in the striatum (DYNB 26% lower; MEAP 39% lower), whereas higher ir levels of N/OFQ (34% higher) were observed. In addition to DYNB, ir N/OFQ and MEAP levels were reduced (23% and 34%, respectively) in the frontal cortex and in the hippocampus a 40% reduction was seen in ir DYNB levels in H rats compared to NH rats.

4. Discussion

We have previously reported increased ir DYN peptide levels in the pituitary gland, hypothalamus, hippocampus, striatum, medulla oblongata and midbrain in male rats after handling (Ploj et al., 1999). In the present study, using female rats, additional regions in the brain were analysed; the frontal cortex, nucleus accumbens, amygdala, substantia nigra, VTA and the PAG. No behavioural evaluation was performed in the present study. However, the procedure followed the same experimental protocol as in a previous study where behavioural effects, using elevated plus-maze, were assessed in male rats (Ploj et al., 1999). This handling procedure has previously been shown to induce long-lasting behavioural effects in both female and male rats with reduced emotionality/anxiety in H rats (Costela et al., 1995; Fernandez-Teruel et al., 1990; Nunez et al., 1995, 1996). Tissue levels of peptides were measured with radioimmunoassays, and the results thus represent steady-state levels of peptides. The alterations in peptides reported here were detected 2 months after the handling procedure. A decrease, for instance, then most likely reflects a reduced synthesis and thereby peptide content, and are here interpreted as a long-term change in neuronal activity.

The results of the present study reveal that neonatal handling induces persistent neurochemical changes in ir peptide levels in the PAG, amygdala and frontal cortex, whereas areas directly related to the hypothalamo–pituitary–adrenal (HPA)-axis were unaffected. The most prominent effects of handling were found in the PAG, where all three peptides were elevated. The midbrain PAG plays a crucial role in the integration of an animal's behavioural, somatic and autonomic responses to threat, stress and pain (Bandler and Shipley, 1994). Handling-induced effects on N/OFQ have to our knowledge not previously been described. A dense plexus of N/OFQ fibers and expression of ORL1 receptors can be found within the PAG (Lachowicz et al., 1995; Schulz et al., 1996). Furthermore, concentrations of N/OFQ precursor mRNA in the PAG are among the highest in the CNS (Houtani et al., 1996), suggesting that N/OFQ might participate in PAG mediated modulation of pain and stressful stimuli. Mice lacking N/OFQ show elevated glucocorticoid levels and display increased anxiety-like behaviour when exposed to a novel and threatening environment (Köster et al., 1999). Furthermore, intracerebroventricular (icv) administration of N/OFQ attenuates behavioural responses to various stressors (Jenck et al., 1997). However, the underlying mechanisms for the anxiolytic effect of N/OFQ are not clear, although PAG has been suggested to be involved (Kyuhou and Gemba, 1999). In addition to N/OFQ, a marked elevation in ir DYNB and ir MEAP levels were detected 2 months after the handling procedure. Several studies have reported the involvement of the opioid system in physiological actions mediated by the PAG. For example, the injection of low doses of morphine into the dorsal PAG increased the relative exploration of the open arms in the elevated plus-maze, indicating an anxiolytic effect of opioid agonists (Brandao, 1993). The finding of high ir levels of N/OFQ in H rats indicates that this peptide system, in addition to the opioid systems, might be of interest to study further with respect to behavioural effects induced by handling (Ader, 1968; Costela et al., 1995; Nunez et al., 1995; Ploj et al., 1999; Wakshlak and Weinstock, 1990).

The PAG connects with several limbic areas including the amygdala, which, like the PAG, has been linked to emotional functions such as anxiety and fear (Davis, 1992). The amygdaloid complex is rich in both opiate receptors and their endogenous ligands (Fallon and Leslie, 1986; Mansour et al., 1988), and changes in enkephalin-degrading peptidase activities in the amygdala has been reported after 15 min daily neonatal handling (days 1–21) (Irazusta et al., 1999). Local injection of an enkephalin analogue (Gallagher et al., 1982) or morphine (File and Rodgers, 1979) into the central nucleus of amygdala interferes with fear and anxiety responses, suggesting an anxiolytic effect of opioids mediated by δ and/or μ receptors. However, opposite effects of opioid peptides in stress responses have been reported. Intracerebroventricular administrations of enkephalin have been reported to attenuate behavioural changes induced by stressful situations, whereas DYN potentiates them (Katoh et al., 1990). The exact anatomical site for these opposing actions is not known. In that respect, it is interesting to note that neonatal handling affects the two opioid peptides analysed here in opposite directions. The pronounced reduction in DYNB levels and, if anything, higher MEAP levels suggest a less active κ-opioid system in H rats, resulting in a dominant δ-opioid system.

Many biochemical modifications in the hippocampus in neonatally H adult rats have been reported. For example,

increased glucocorticoid receptor expression has been seen in the hippocampus in adult rats after 15 min daily handling during the first 3 weeks of life. The difference in hippocampal glucocorticoid receptor levels appears to be related to the decreased HPA responsivity to stress in H animals (Meaney et al., 1996). The hippocampus is densely innervated by dynorphinergic neurons (Henriksen et al., 1982), and the expression of proDYN in male rats has been shown dependent on glucocorticoid activity. Excess of glucocorticoids elevates DYN ir, whereas adrenalectomy reduces DYN ir and proDYN mRNA (Thai et al., 1992). We have previously reported increased levels of ir DYN peptides in male H rats, which may be interpreted as a consequence of an increased glucocorticoid receptor expression (Ploj et al., 1999). In this study, the ir DYNB levels were not increased, they were instead lower in H rats, although the reduction was not significantly different from NH rats. Handling thus affects the DYN system differently depending on the gender of the pup. Although both male and female rats have increased glucocorticoid receptor expression in the hippocampus after handling (Meaney et al., 1991), important sex differences in HPA responses to stress (Brett et al., 1986; Meaney et al., 1991) and in pituitary–adrenal activity (Critchlow et al., 1963; Kitay, 1961) have been reported. In addition, several reports suggest that male and female rats and mice differ with respect to endogenous opioid responses to stress (Baamonde et al., 1989; Farabollini et al., 1991; Fernandez et al., 1999; Klein et al., 1998; Mogil and Belknap, 1997). Our present and previous results show that handling also induces gender-specific effects in the hippocampus, an increase of ir DYN in male rats and no statistically significant change in female rats.

Except from the hippocampus, 15 min daily handling until weaning has been reported to induce changes in glucocorticoid receptor expression in the frontal cortex in male rats. (Bhatnagar and Meaney, 1995). Here, we observed low levels of all three peptides in H rats, although only the reduction in ir DYNB levels was statistically different from NH rats. The frontal cortex is a region of the limbic system that has been suggested to be involved in the regulation of HPA activity (Diorio et al., 1993; Feldman and Conforti, 1985) and is also a sensory-motor integration region involved in affective processing (Kolb, 1984). Previous reports, using receptor-binding techniques, have shown profound effects on the μ- and δ-opioid systems after handling-induced stress. The effects of gentle handling on opioid systems were even more pronounced and affected more regions than foot-shock (Stein et al., 1992). The low ir DYNB levels detected here in the H rats may reflect a role for the κ-opioid system in the altered emotional processing in these rats. Reduced ir levels of DYNA (1–13) have previously been shown in the frontal cortex acutely after foot-shock stress (Millan et al., 1981), but long-term stress-induced effects have not been elucidated.

We have previously reported increased ir levels of DYNA and DYNB in the pituitary and the hypothalamus in H male rats (Ploj et al., 1999). The significance of increased ir DYN levels has however been difficult to establish. The HPA-axis is clearly affected by handling as evidenced by a reduced response to stress (Meaney et al., 1996). DYN 1–8 (Roth et al., 1983) and enkephalin (Hökfelt et al., 1983) are colocalised with CRH in neurosecretory neurons originating in the hypothalamus and endogenous opioids have been suggested to regulate the activity of the HPA-axis. The exact mechanism has however been difficult to elucidate and a mixed picture of either inhibition or facilitation has been presented (Calogero et al., 1996; Iyengar et al., 1986; Plotsky, 1986). In this study, we did not see any significant changes in either ir DYNB or ir MEAP levels. The DYN system thus seems to be affected differently in male and female rats. These results add to the previously reported sex differences in HPA responses to stress (Brett et al., 1986; Meaney et al., 1991) and in pituitary–adrenal activity (Critchlow et al., 1963; Kitay, 1961).

This is the first study using female rats that examines effects of neonatal handling on opioid and N/OFQ peptides. Our choice was to investigate possible sex-dependent handling-induced effects in several sequential steps, and the main question in this study was to examine whether neonatal handling in female rats could induce similar changes in peptide levels as previously described in male rats (Ploj et al., 1999). In the present paper, we present evidence that neonatal handling indeed induce long-term changes in peptide levels in female rats, but different from those described in male rats. In this study, we cannot rule out the possibility that endogenous levels of oestrogen and progesterone cause the observed sex differences. The next step is to examine the origin of the reported sex dependent effects and further handling experiments including comparisons of intact females in different phases of the oestrous cycle and ovariectomized rats are now needed to address the influence of gonadal hormones.

In conclusion, neonatal handling was found to induce long-term changes in N/OFQ and opioid peptide systems in the PAG and in ir DYN levels in the amygdala and frontal cortex in female rats. In contrast to earlier findings in male rats, no changes were detected in areas related to the HPA-axis in female rats. The results show that an environmental change early in life, such as handling during the first 3 weeks of life, is sufficient to induce persistent changes in neuropeptide systems. Furthermore, these changes may be involved in the previously described behavioural consequences of handling and indicate a possible role for the N/OFQ and the opioid system in the emotional/motivational aspects of mild chronic stress induced by handling.

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