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Effects of maternal separation on voluntary ethanol intake and brain peptide systems in female Wistar rats

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Abstract

In previous studies, changes in adult ethanol intake after early environmental experiences, such as short and prolonged maternal separation, have been described in male rats. The aim of this study was to further investigate long-term effects of maternal separation on voluntary ethanol intake as well as brain opioid and nociceptin/orphanin FQ (N/OFQ) peptides in female Wistar rats. During postnatal days (PNDs) 1–21, rat pups were subjected to 15 min (MS15) or 360 min (MS360) of daily maternal separation, or were kept under normal animal facility rearing (AFR) conditions. At 10 weeks of age, ethanol intake was measured using a two-bottle free choice paradigm and dynorphin B (DYNB), Met-enkephalin-Arg^{6}-Phe^{7} (MEAP) and N/OFQ levels were analyzed. MS15 resulted in changes in hypothalamus (DYNB), medial prefrontal cortex and nucleus accumbens (MEAP), and amygdala (N/OFQ). MS360 induced alterations in medial prefrontal cortex (MEAP) and amygdala (N/OFQ). MS15 and MS360 had no effect on ethanol intake. However, 4 weeks of ethanol intake affected peptide levels differently in MS15, MS360 and AFR rats and resulted in attenuation of the separation-induced differences. These results show that even though maternal separation has no effect on voluntary ethanol intake in female rats, the ethanol-induced effects on peptide levels depend on the early environmental setting.
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Keywords: Handling; Maternal deprivation; Opioid; Nociceptin/orphanin FQ; Alcohol

1. Introduction

It is generally accepted that early environmental experiences have profound influences on brain development and on adult brain function and behavior. There is evidence that prenatal as well as postnatal adverse experiences can disturb the normal brain development and cause neurobiological and behavioral changes (Anand and Scalzo, 2000; Andersen, 2003). Early traumatic events, such as maternal stress or illness, postnatal neglect and/or abuse, may also contribute to the vulnerability for psychopathology and/or drug dependence in adulthood, both in animals (Gordon, 2002; Newport et al., 2002; Sanchez et al., 2001) and in humans (Gilmer and McKinney, 2003; Kendler et al., 2000; Langeland et al., 2004; Spak et al., 1998). Environmental factors play an important role in alcohol abuse, and these factors interact to a large degree with genetic influences (Cloninger et al., 1981; Gianoulakis, 2001). In both human and animal studies it has been shown that the individual vulnerability to develop alcohol dependence involves a strong genetic variable (Crabbe and Phillips, 2004; Dick and Foroud, 2003; Schumann et al., 2003) and further studies on environmental factors as protective or risk factors are now of great interest.

The neurochemical mechanisms underlying the genetic and environmental influences on brain function are not fully elucidated. The maternal separation paradigm can be used as an experimental model for studies of mechanisms underlying the impact of early environmental factors on behavior later in life. The offspring is separated from the mother, either in litters or individually, during the pre-weaning period and the consequences can be assessed in the adult animal (Ladd et al., 2000; Lehmann and Feldon, 2000; Pryce and Feldon, 2003). Maternal separation for short time

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periods (3–20 min), also referred to as handling, is often associated with behavioral effects such as decreased stress response in the adult offspring. Prolonged separation periods (>1 h), on the other hand, seem to affect these parameters differently, sometimes with an opposite outcome (Anisman et al., 1998; Ladd et al., 2000; Newport et al., 2002). Maternal separation can also affect the propensity to drink ethanol later in life. A lower voluntary ethanol intake has been described after short separation periods (Hilakivi-Clarke et al., 1991; Ploj et al., 2003a; Roman et al., 2003; Weinberg, 1987), while prolonged maternal separation enhanced the ethanol intake (Huot et al., 2001; Ploj et al., 2003a; Roman et al., 2005) The maternal separation procedure thus seem suitable to serve as a model for further studies on mechanisms underlying early environmental influence on ethanol intake. In a series of experiments, we currently examine how maternal separation affects neurochemistry, behavior and voluntary ethanol intake later in life.

Examples of interesting endogenous target systems for further studies of mechanisms underlying environmental protective and risk factors for the propensity to develop ethanol dependence are the opioid and nociceptin/orphanin FQ (N/OFQ) systems. The opioid peptides derive from proopiomelanocortin, proenkephalin and prodynorphin, and act at the μ-, δ- and κ-receptors, respectively (Akil et al., 1998; Terenius, 2000). The opioid peptide system is involved in a variety of physiological functions, such as pain modulation, reward and motivation (Herz, 1998; Nylander and Silberring, 1998; Terenius, 1992). The role for the opioid systems in ethanol reinforcement and addiction has extensively been studied (Gianoulakis, 2004; Oswald and Wand, 2004; Van Ree et al., 1999). Genetically modified strains of mice lacking one or more opioid receptor genes are currently used to further study the involvement of the opioid systems in responses to behavior, stress and drugs of abuse (Gerrits et al., 2003; Kieffer and Gaveriaux-Ruff, 2002). Differences in the dynorphin (DYN) and enkephalin systems have been described between genetically divergent ethanol-preferring (Lewis, AA) and ethanol-avoiding (Fischer, ANA) rats (Nylander et al., 1994, 1995). In addition, differences in opioid systems were detected in C57BL/6J and DBA/2J mice with a high and low ethanol preference, respectively (Jamensky and Gianoulakis, 1997, 1999; Ploj et al., 2000). N/OFQ is the endogenous ligand for the opioid receptor like-1 (ORL1) receptor (Bunzow et al., 1994; Meunier et al., 1995; Mollereau et al., 1994; Reinscheid et al., 1995). N/OFQ and its receptor are widely distributed in brain areas involved in reward processes. It may act as a functional antiopioid peptide in the control of brain nociceptive processes, as well as affecting other behaviors such as locomotion, feeding and anxiety (Ciccocioppo et al., 2000; Harrison and Grandy, 2000). An activation of the N/OFQ system also can dampen the rewarding effects of ethanol, morphine and cocaine, as well as reduce reinstatement of stress-induced alcohol-seeking behavior (Ciccocioppo et al., 2003).

Recent results, using maternal separation in male rats, indicate that early environmental influences can induce neurochemical changes in the endogenous opioid and N/OFQ systems (Ploj and Nylander, 2003; Ploj et al., 1999, 2002, 2003a,b). However, most studies have been performed in male rats and the aim of this study was to further examine the outcome of maternal separation in female rats. The use of females in experimental studies has become an important issue, since it has been demonstrated that there are differences between females and males in many health outcomes, including pain sensitivity, stress response, immune function, pathological diseases and mood disorders (Klein and Corwin, 2002). In addition, sex differences are observed in the actions of ethanol on the central nervous system at the molecular, cellular as well as the behavioral level, involving a variety of neurotransmitter systems (Devaud et al., 2003). Sex dependent consequences of maternal separation have previously been shown (Kalinichev et al., 2002; Lehmann et al., 1999; McIntosh et al., 1999; Sluyter et al., 2000; Wigger and Neumann, 1999). In the current study, we investigated the long-term effects of short (15 min; MS15) and prolonged (360 min; MS360) periods of daily maternal separation on voluntary ethanol intake in female Wistar rats. In addition, we analyzed separation-induced effects on immunoreactive (ir) DYNB, Met-enkephalin-Arg^6-Phe^7 (MEAP) and N/OFQ levels. Furthermore, we address the question whether voluntary ethanol intake may affect peptide levels differently dependent on early environmental setting, and therefore compared ethanol-induced effects in rats subjected to MS15 and MS360, respectively, with animal facility reared (AFR) rats.

2. Materials and methods

2.1. Animals

Time-mated pregnant Wistar rats (Scanbur BK AB, Sollentuna, Sweden) arrived at the animal department on gestation days 13–15. Upon arrival, the dams were singly housed in standard macrolon cages (59 × 38 × 20 cm) containing wood-chip bedding material and nesting material, and were maintained on pellet food (R36 Labfor; Lactamin, Vadstena, Sweden) and water ad libitum. All animals were housed in a temperature- (22 ± 1 °C) and humidity- (50 ± 10%) controlled animal room on a 12-h light/dark cycle with lights on at 06:00 h. All animal experiments were performed under a protocol approved by the Uppsala animal ethical committee and in accordance with the Swedish Animal Protection Legislation.

2.2. Maternal separation

On the day of birth (day 0), the litters were sexed and cross-fostered into 10 pups per litter, although in a small

number of litters this was not possible, with the same composition of female and male pups. This arrangement was used to avoid the use of littermates in the experimental groups. The litters were then randomly assigned to one of three rearing conditions; either 15 min (MS15, $n=8$) or 360 min (MS360, $n=8$) of daily maternal separation, or housed under normal animal facility rearing (AFR, $n=8$). The separations occurred once daily during postnatal days (PNDs) 1–21. First the dam and then the pups were removed from the nest. Each litter was placed in macrolon cages (26 × 20 × 14 cm) containing wood chip bedding material and moved to an adjacent room (27 ± 1 °C). These cages were changed everyday. Separation sessions were performed during the light period, 15 min for the MS15 with the first litter starting at 09:00 h and 360 min for the MS360 starting at 09:30 h. Only three persons were allowed to enter the room and perform all separations and care giving of the rats. During the separations, the dams in the MS15 groups were moved to other cages and the litters were returned before the dams. In the MS360 groups, the dams were returned to the home cages during the separations, and moved again before the litters were returned. Home cages were changed two times during PNDs 1–21 for all three groups, with old bedding material mixed with clean bedding material. The MS15 and MS360 litters were weighed every third day on PNDs 1, 4, 7, 10, 13, 16 and 19. The AFR litters were weighed on the same days as the cages were changed on PNDs 7 and 16, to disturb them as little as possible. All litters were also weighed on the day for weaning on PND 22. The animals were thereafter housed in the same treatment groups with 5 males or 5 females per cage (59 × 38 × 20 cm). Only female rats were used in this experiment. On PNDs 68–69, after 7 weeks under normal animal facility care, with cage changes once a week, 30 female rats (MS15, $n=10$; MS360, $n=10$; AFR, $n=10$) were randomly selected, weighed and then sacrificed by decapitation and used for analysis of ir peptide levels. Before decapitation each rat was singly housed for 30 min in order to control for conditions regarding the decapitation. The remaining 86 female rats (MS15, $n=28$; MS360, $n=29$; AFR, $n=29$) were used for the studies on voluntary ethanol intake. Furthermore, rats from all three experimental groups were randomly selected (MS15, $n=9$–14; MS360, $n=11$–15; AFR, $n=10$–14) for analysis of ir peptide levels after the period with voluntary ethanol intake.

2.3. Voluntary ethanol intake

Seven weeks after the separation treatments, on PNDs 70–73, the rats ($n=86$) were singly placed in cages (42 × 26 × 18 cm) and were introduced to ethanol in a two-bottle free choice paradigm. The rats were given access to one bottle with water and one bottle with ethanol solution in gradually increasing concentrations (v/v) from 2% to 8% during a period of 31 days (4 days with 2%, 4 days with 4%, 14 days with 6% and finally 9 days with 8%). Bottle positions were changed everyday to avoid position preference. Ethanol and water intakes were measured everyday, while food intake and body weight were measured every third day. All measurements were made in the afternoon. To examine the effects of voluntary ethanol intake on peptide levels in MS15 and MS360 animals in comparison to AFR animals, the rats were decapitated on PNDs 103–104, after 31 days with a free choice between ethanol and water, and the brains were dissected for ir peptide analysis.

2.4. Dissection

After decapitation, the brain and the pituitary gland were taken out. The pituitary gland was divided into the anterior and neurointermediate lobes. The hypothalamus was removed from the brain and thereafter the brain was placed in a cooled brain matrix (AgnTho's AB; Stockholm, Sweden) and sliced manually with razor blades in coronal sections. The following 9 structures were dissected: frontal cortex, medial prefrontal cortex, nucleus accumbens, striatum, hippocampus, amygdala, substantia nigra, ventral tegmental area and periaqueductal gray. The tissues were immediately frozen on dry ice and stored at −80 °C until further analysis.

2.5. Tissue homogenization, extraction and separation of peptides

The tissue extraction was performed with heated 1 M acetic acid. The tissues were heated for 5 min at 95 °C, cooled on ice and homogenized by sonication using a Branson Sonifier and then reheated for 5 min at 95 °C. After cooling on ice, the samples were centrifuged for 15 min at 12,000×g in a Beckman GS-15R Centrifuge. The supernatants were purified using a cation exchange procedure (Christensson-Nylander et al., 1985; Ploj et al., 2000). The samples were added onto small columns containing Sephadex C-25 gel (Pharmacia Diagnostics, Uppsala, Sweden), and the opioid and N/OFQ peptides were stepwise eluted in separate fractions using buffers containing a mixture of purified pyridine and formic acid with increasing ion strength. Thereafter the fractions were dried in a vacuum centrifuge and stored at −20 °C.

2.6. Radioimmunoassays

The peptides were analyzed using specific radioimmunoassays (RIAs) according to methods previously described in detail for DYNB and MEAP (Nylander et al., 1995, 1997) and for N/OFQ (Ploj et al., 2000). The tracer peptides were labelled using chloramin-T and purified with high-performance liquid chromatography. In the N/OFQ assay, Tyr^{14}-N/OFQ was used as the tracer peptide. The samples and standards for each peptide were dissolved in methanol/0.1 M hydrochloric acid (1:1). Samples subjected to MEAP assay were oxidized prior to the RIA procedure. Samples

were dissolved in 100 μl 1 M acetic acid, to which 10 μl of 30% H_2O_2 was added. They were then incubated at 37 °C for 30 min and dried in a vacuum centrifuge. An aliquot of either 25 μl of the samples or respective standard peptide was incubated with 100 μl of antiserum and 100 μl of ^{125}I-labelled peptide for 24 h at 4 °C. Antiserum for the respective peptide was generated in rabbits and used in a final dilution of: DYNB (113+) 1:742,500, MEAP (90:3D II) 1:135,000, and N/OFQ (96:2+) 1:112,500, respectively. 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 dynorphin (DYN 32) 100%. Other opioid peptides did not cross-react with the DYNB antiserum. Cross-reactivity for the MEAP antiserum with Met-enkephalin, Met-enkephalin-Arg^6, Met-enkephalin-Arg^{6}Gly^{7}Leu^8, Leu-enkephalin and DYNA (1–6) was less than 0.1%. The 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% (Christensson-Nylander et al., 1985; Ploj et al., 2000). The DYNB and N/OFQ antiserum and labelled peptide were diluted in a gelatine buffer containing 0.15 M sodium chloride, 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 MEAP gelatine buffer contained 0.15 M sodium chloride, 0.025 M EDTA, 0.1% gelatine and 0.1% bovine serum albumin in a 0.05 M sodium phosphate buffer.

To separate free and antibody bound peptides in the DYNB and N/OFQ assays, respectively, 100 μl of a sheep anti-rabbit antiserum (Pharmacia Decanting Suspension, 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* in a cooled Beckman GS-15R Centrifuge, the supernatant was discarded and the radioactivity in the pellet was counted in a Wallac 1470 Wizard gamma counter. For MEAP, 200 μl of a charcoal suspension, 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 1 min at 12,000×*g*. The radioactivity in a 300 μl aliquot of the supernatant was measured in the gamma counter.

2.7. Statistical analyses

Differences between the three experimental groups, MS15, MS360 and AFR, respectively, in body weights and in peptide levels were analyzed using the parametric one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference (PLSD) post hoc test. The fluid intake and the food intake were analyzed with non-parametric statistics, Kruskal–Wallis followed, where significant, by pairwise Mann–Whitney *U*-test comparisons, since neither of these data showed a normal distribution. The statistically significant effects found with Mann–Whitney *U*-test were further corrected using the Bonferroni–Holm correction (Holm, 1979), in order to correct for type I errors. To correlate ethanol intake and peptide levels the non-parametric Spearman Rank Correlation was used. All statistical analyses were performed using StatView 5.0.1 (SAS Institute Inc., Cary, NC) computer software for Macintosh computers. Differences were considered significant at $p<0.05$.

3. Results

3.1. Effects on body weight

There were no differences in mean pup weight (g) between the experimental groups during PNDs 1–21 or at the time for weaning on PND 22 (Fig. 1). Furthermore, no differences were found between body weights in the experimental groups at 10 weeks of age. In the rats used for neurochemical analysis, the mean ± standard error of the mean (S.E.M.) body weights were 230 ± 5.5 g (MS15), 222 ± 6.8 g (MS360) and 217 ± 6.4 g (AFR), respectively, on the day for decapitation (PNDs 68–69). In the rats used for ethanol intake measurements, the body weights were 231 ± 4.1 g (MS15), 228 ± 3.6 g (MS360) and 219 ± 2.8 g (AFR), respectively, on the first day of access to ethanol (PNDs 70–73). During the period with voluntary ethanol intake there were only a few occasional statistically significant differences between the three experimental groups. The MS15 rats had a higher mean body weight than the AFR rats at 2 out of 11 occasions ($p<0.05$), whereas the MS360 rats had a higher mean body weight than the AFR rats at 1 out of 11 occasions ($p<0.05$). The last measurement, on the day for decapitation on PNDs 103–104, the mean body weight differed between the three groups [$F(2,83)=3.24$, $p<0.05$], with a higher body weight in the MS15 group (274 ± 4.7 g) compared to the AFR group (259 ± 3.6 g; $p<0.05$). The mean body weight in the MS360 group was 271 ± 4.7 g.

3.2. Effects on fluid intake

The daily ethanol intake patterns (g/kg/day) in MS15, MS360 and AFR rats, respectively, during continuous access to ethanol are shown in Fig. 2. The median daily ethanol intake was similar during the entire period in the three experimental groups.

In Table 1, the ethanol intake at the different ethanol concentrations is shown. The statistical analysis showed no differences between the experimental groups. When analyzing the number of animals with a high (≥ 1.00 g/kg/day) and low (<1.00 g/kg/day) ethanol intake, respectively, there were a slight, but not significant, tendency for fewer animals with an ethanol intake above 1.00 g/kg/day in the MS15 group compared to the MS360 and AFR groups (Fig. 3).

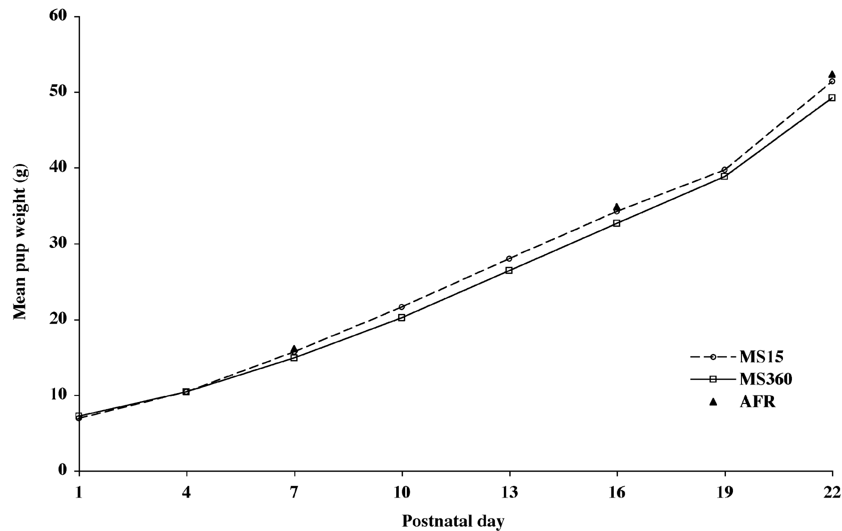

Fig. 1. The mean pup weight (g) during postnatal days 1–22 in litters exposed to 15 min (MS15, $n=8$) or 360 min (MS360, $n=8$) of daily maternal separation, or kept under normal animal facility rearing (AFR, $n=8$) conditions. Data are expressed as mean ± standard error of the mean (S.E.M.).

No statistically significant differences were detected in ethanol preference (% of total fluid intake), total fluid intake (g/kg/day) or in water intake (g/kg/day) between the experimental groups during the period with access to ethanol (data not shown).

3.3. Food intake

There were no major effects of maternal separation on food intake during the period with access to ethanol (data not shown). Differences were found between the experimental groups at the first out of 10 measurements [$H=7.14$, $p<0.05$], with a significantly lower food intake in the MS360 animals than in the AFR animals [$U=249.5$, $p<0.05$].

3.4. Separation-induced effects on ir peptide levels

The mean ± S.E.M. ir peptide levels in various brain areas of MS15, MS360 and AFR rats at 10 weeks of age are illustrated in Table 2A–C. In the MS15 rats, significantly lower ir DYNB levels were found in the hypothalamus compared to both the MS360 and the AFR rats (Table 2A). In the other brain areas and the pituitary gland, similar ir DYNB peptide levels were observed in the three experimental groups.

The ir MEAP levels in MS15, MS360 and AFR rats at 10 weeks of age are shown in Table 2B. In the medial prefrontal cortex, the MS15 and MS360 rats showed statistically significant higher ir MEAP levels than the AFR rats. In addition, in the nucleus accumbens the MS15 rats had higher

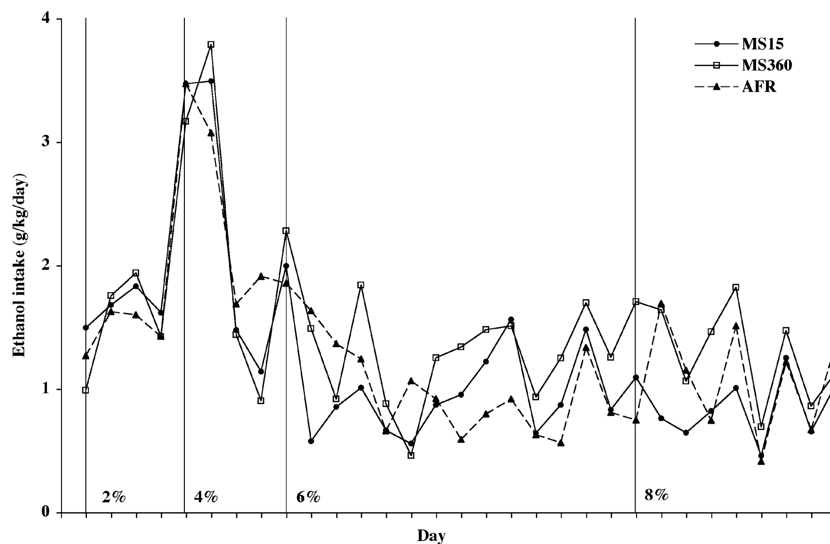

Fig. 2. The median daily ethanol intake patterns (g/kg/day) during the entire period with voluntary ethanol intake in rats exposed to 15 min (MS15, $n=28$) or 360 min (MS360, $n=29$) of daily maternal separation, or kept under normal animal facility rearing (AFR, $n=29$) conditions during postnatal days 1–21. The ethanol period with continuous access to gradually increasing concentrations of ethanol started on postnatal days 70–73.

Table 1
Effects of maternal separation on voluntary ethanol intake in female Wistar rats

Ethanol concentration (%)	MS15	MS360	AFR
2	1.71 ± 0.32	1.37 ± 0.37	1.47 ± 0.39
4	2.78 ± 0.93	2.29 ± 1.01	2.21 ± 1.01
6	1.22 ± 0.60	1.66 ± 0.76	1.32 ± 0.90
8	0.89 ± 0.43	1.41 ± 0.70	1.33 ± 0.87

The median ± median absolute deviation (M.A.D.) ethanol intake (g/kg/day) during the different ethanol concentrations in rats exposed to 15 min (MS15, $n = 28$) or 360 min (MS360, $n = 29$) of daily maternal separation, or kept under normal animal facility rearing (AFR, $n = 29$) conditions during postnatal days 1–21.

ir MEAP levels than the AFR rats. Ir MEAP levels were not detectable in the pituitary gland. In the other brain areas, no differences in ir MEAP levels were found between the experimental groups.

In the amygdala, both the MS15 and MS360 rats showed statistically significant lower ir N/OFQ levels than the AFR rats (Table 2C). N/OFQ was not analyzed in the pituitary gland. In all other brain areas the ir N/OFQ levels were similar in the experimental groups.

3.5. Ethanol-induced effects on ir peptide levels in MS15, MS360 and AFR rats

The effect of voluntary intake on ir peptide levels in MS15, MS360 and AFR rats, respectively, are shown in Table 2A–C. To illustrate possible differences in ethanol-induced effects between the three experimental groups, the ir peptide levels after the 4-week period with access to ethanol were compared with the ir peptide levels prior to ethanol exposure. The ir peptide levels after voluntary ethanol intake, expressed as percentage of ir peptide levels in the corresponding group of animals not exposed to ethanol, are shown within parenthesis in Table 2A–C.

In the neurointermediate pituitary lobe, the MS15 rats had significantly lower ir DYNB levels than both the MS360 and the AFR rats (Table 2A). In the frontal cortex, the MS360 rats had significantly higher ir DYNB levels compared to the AFR rats. In these areas, no differences were observed prior to the ethanol-drinking period. No differences were detected in the hypothalamus, an area in which separation-induced effects were observed. In all other brain areas no differences were detected.

Ir MEAP levels were higher in the hypothalamus in the MS15 group compared to both the MS360 and AFR groups (Table 2B). In this brain area, the ir MEAP levels were similar in the three groups prior to ethanol intake. In the medial prefrontal cortex and nucleus accumbens, where differences between the experimental groups were detected prior to ethanol exposure, no differences were found. In all other brain areas the peptide levels were similar in the experimental groups.

In the medial prefrontal cortex, the MS360 group showed significantly higher ir N/OFQ levels than the AFR group (Table 2C). In the other brain areas there were no differences between the experimental groups. In the amygdala, ir N/OFQ levels were affected by maternal separation but after voluntary ethanol intake no differences were detected between experimental groups.

3.6. Correlation between ethanol intake and peptide levels

The correlation between the mean ethanol intake during the period with 8% ethanol solution and ir DYNB, MEAP and N/OFQ peptide levels, respectively, in the different brain areas were evaluated. No correlation between voluntary ethanol intake and ir DYNB peptide levels was found in any of the analyzed brain areas in the three experimental groups. Within the AFR group, a positive correlation was found in the medial prefrontal cortex, i.e. elevated ir MEAP

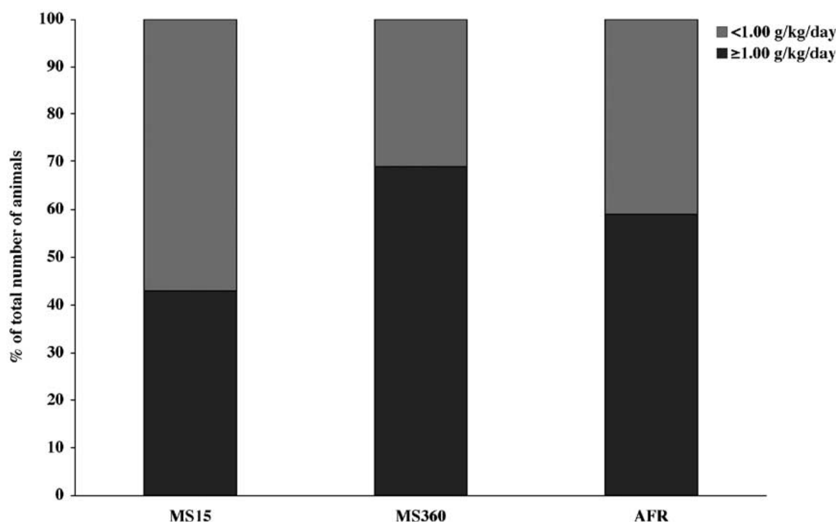

Fig. 3. Percentage of the total number of animals with an ethanol intake below or above 1.00 g/kg/day, respectively, at 8% ethanol, in rats exposed to 15 min (MS15, $n = 28$) or 360 min (MS360, $n = 29$) of daily maternal separation, or kept under normal animal facility rearing (AFR, $n = 29$) conditions during postnatal days 1–21.

Table 2
Immunoreactive peptide levels, after separation at 10 weeks of age (S; $n = 8-10$) as well as after 4 weeks of voluntary ethanol intake (E; $n = 9-15$), in rats exposed to 15 min (MS15) or 360 min (MS360) of daily maternal separation, or kept under normal animal facility rearing (AFR) conditions during postnatal days 1–21

Brain area		MS15	MS360	AFR
(A) Immunoreactive dynorphin B (DYNB) levels				
AL	S	3.31±0.31	3.10±0.23	3.10±0.41
	E	5.13±0.38	5.07±0.67	4.03±0.33
		(+55%)	(+63%)	(+30%)
NIL	S	135.89±7.09	126.00±4.78	117.86±5.74
	E	175.80±10.83**#	212.14±9.01	230.72±16.63
		(+29%)	(+67%)	(+96%)
HT	S	23.60±1.61*#	29.48±1.37	29.04±1.52
	E	27.94±1.54	26.11±1.72	26.74±1.29
		(+18%)	(−11%)	(−8%)
FCX	S	2.23±0.25	2.45±0.14	2.38±0.13
	E	2.18±0.27	2.73±0.17**	1.76±0.26
		(−2%)	(+12%)	(−26%)
MPFCX	S	1.56±0.08	1.41±0.10	1.48±0.13
	E	0.97±0.06	1.03±0.04	0.96±0.05
		(−38%)	(−26%)	(−35%)
NAC	S	29.40±3.68	27.85±2.09	23.21±6.72
	E	14.89±1.49	14.23±0.94	15.84±1.25
		(−49%)	(−49%)	(−32%)
STR	S	14.74±0.59	14.85±0.87	13.28±0.71
	E	14.04±0.53	12.96±0.53	12.18±0.54
		(−5%)	(−13%)	(−8%)
HC	S	13.81±0.51	13.81±0.82	14.14±0.76
	E	17.95±0.87	18.15±0.90	17.67±0.80
		(+30%)	(+31%)	(+25%)
AMY	S	4.10±0.33	4.07±0.24	4.50±0.52
	E	2.69±0.21	2.61±0.17	2.54±0.18
		(−34%)	(−36%)	(−44%)
SN	S	22.80±1.74	28.36±3.13	23.85±3.37
	E	25.77±2.34	25.59±2.16	25.19±1.76
		(+13%)	(−10%)	(+6%)
VTA	S	1.78±0.27	1.82±0.12	1.64±0.29
	E	1.09±0.14	0.92±0.10	1.06±0.14
		(−39%)	(−49%)	(−35%)
PAG	S	4.07±0.44	4.62±0.66	4.05±0.36
	E	3.92±0.20	4.28±0.34	4.58±0.35
		(−4%)	(−7%)	(+13%)
(B) Immunoreactive Met-enkephalin-Arg^6-Phe^7 (MEAP) levels				
AL		n.d.	n.d.	n.d.
NIL		n.d.	n.d.	n.d.
HT	S	61.27±4.43	62.22±2.87	66.22±4.20
	E	74.75±2.61**##	63.90±2.35	63.33±3.42
		(+22%)	(+3%)	(−4%)
FCX	S	5.73±0.72	5.29±0.49	5.67±0.56
	E	8.23±0.86	6.81±0.63	7.77±0.68
		(+44%)	(+29%)	(+37%)
MPFCX	S	2.82±0.18**	2.56±0.21*	1.70±0.32
	E	4.43±0.59	3.94±1.23	3.35±0.33
		(+57%)	(+54%)	(+97%)
NAC	S	45.36±6.65**	36.07±4.82	24.89±3.50
	E	63.60±4.92	59.22±3.33	63.88±2.23
		(+40%)	(+64%)	(+157%)
STR	S	62.86±3.19	65.82±2.87	57.92±3.99
	E	71.76±3.26	70.25±2.85	68.01±3.17
		(+14%)	(+7%)	(+17%)

Table 2 (*continued*)

Brain area		MS15	MS360	AFR
(B) Immunoreactive Met-enkephalin-Arg^6-Phe^7 (MEAP) levels				
HC	S	6.14±0.33	6.48±0.42	5.80±0.40
	E	7.47±0.48	7.98±0.39	7.55±0.38
		(+22%)	(+23%)	(+30%)
AMY	S	20.45±2.20	21.63±2.90	21.07±1.95
	E	19.75±1.47	20.22±1.16	17.69±1.57
		(−3%)	(−7%)	(−16%)
SN	S	0.70±0.07	0.87±0.10	0.66±0.09
	E	2.09±0.19	2,87±0.53	2.77±0.40
		(+199%)	(+230%)	(+320%)
VTA	S	2.79±0.27	2.89±0.29	2.99±0.53
	E	4.85±0.66	4.65±0.52	5.80±1.00
		(+74%)	(+61%)	(+94%)
PAG	S	13.82±2.95	17.97±3.50	15.49±1.28
	E	29.55±1.94	29.44±1.68	28.84±2.51
		(+114%)	(+64%)	(+86%)
(C) Immunoreactive nociceptin/orphanin FQ (N/OFQ) levels				
HT	S	12.92±0.42	13.87±0.42	13.82±0.46
	E	13.52±0.49	13.55±0.30	13.73±0.46
		(+5%)	(−2%)	(−1%)
FCX	S	2.33±0.18	2.51±0.12	2.74±0.19
	E	2.24±0.17	2.28±0.13	1.79±0.18
		(−4%)	(−9%)	(−35%)
MPFCX	S	2.02±0.11	1.84±0.16	1.83±0.18
	E	0.81±0.09	0.96±0.11**	0.56±0.06
		(−60%)	(−48%)	(−69%)
NAC	S	2.30±0.15	2.41±0.14	2.20±0.13
	E	1.73±0.23	1.30±0.10	1.55±0.15
		(−25%)	(−46%)	(−30%)
STR	S	1.06±0.11	1.05±0.04	1.16±0.06
	E	1.00±0.06	0.90±0.05	0.84±0.03
		(−6%)	(−14%)	(−28%)
HC	S	3.12±0.08	2.91±0.14	3.13±0.14
	E	3.14±0.08	3.05±0.09	2.97±0.09
		(+1%)	(+5%)	(−5%)
AMY	S	4.44±0.19*	4.02±0.23**	5.18±0.30
	E	2.55±0.21	2.83±0.21	2.81±0.24
		(−43%)	(−30%)	(−46%)
SN	S	0.77±0.14	0.91±0.14	0.75±0.09
	E	0.53±0.10	0.53±0.07	0.42±0.06
		(−31%)	(−42%)	(−44%)
VTA	S	1.60±0.20	1.67±0.22	1.78±0.33
	E	0.99±0.11	0.98±0.09	0.96±0.14
		(−38%)	(−41%)	(−46%)
PAG	S	5.06±0.57	5.44±0.50	6.08±0.62
	E	6.17±0.39	5.87±0.39	6.49±0.32
		(+22%)	(+8%)	(+7%)

The peptide levels represent mean±standard error of the mean (S.E.M.) and are expressed as fmol/mg tissue. The value within parenthesis shows the ratio (expressed as percent higher or lower) of peptide levels after the ethanol intake period as compared to peptide levels in rats not exposed to ethanol in the MS15, MS360 and AFR groups, respectively. Anterior pituitary lobe (AL); neurointermediate pituitary lobe (NIL); hypothalamus (HT); frontal cortex (FCX); medial prefrontal cortex (MPFCX); nucleus accumbens (NAC); striatum (STR); hippocampus (HC); amygdala (AMY); substantia nigra (SN); ventral tegmental area (VTA); periaqueductal gray (PAG); not detectable (n.d.). *$p < 0.05$, **$p < 0.01$ compared to AFR rats; #$p < 0.05$, ##$p < 0.01$ compared to MS360 rats (one-way ANOVA, Fisher's PLSD post hoc test).

levels were seen with an increased ethanol intake ($r=0.56$, $p<0.05$) whereas in the substantia nigra, a negative correlation was observed ($r=-0.67$, $p<0.05$). Within the MS360 group, a high ethanol intake correlated with low ir MEAP levels ($r=-0.79$, $p<0.01$) in the periaqueductal gray and low ir N/OFQ peptide levels ($r=-0.53$, $p<0.05$) in the amygdala.

4. Discussion

There is an increasing interest in sex differences in the vulnerability and outcome of drug abuse. Socio-cultural factors and/or innate biological factors can contribute to differences in patterns of drug use and abuse (Lynch et al., 2002) and in animal models, females seem to be more sensitive to the rewarding effects of drugs, from the initial acquisition of drug self-administration, to maintenance, escalation and relapse (Carroll et al., 2004). In recent studies, using the same protocol as herein, sex differences have been reported in separation-induced effects on ethanol intake. MS15 and MS360 had no effect on voluntary ethanol intake in female Wistar rats (Roman et al., 2004) whereas male Wistar rats had a low ethanol intake after MS15 and a high intake after MS360 (Ploj et al., 2003a). The present study, which comprises a more detailed analysis of the consequences of maternal separation on ethanol intake in female rats, confirms that neither MS15 nor MS360 has a major effect on voluntary ethanol intake in female Wistar rats. However, there was a tendency for fewer animals in the MS15 group with an intake above 1.00 g/kg/day during the period with 8% ethanol solution. Short periods of maternal separation may thus constitute a protective factor for high ethanol intake in adulthood, but in female rats this protection is not as pronounced as for male rats (Hilakivi-Clarke et al., 1991; Ploj et al., 2003a; Weinberg, 1987). In the previous male Wistar rat study, a trend towards high and low responders among the animals in the MS360 group was observed (Ploj et al., 2003a). In the present study, no clear responders or non-responders in the MS360 group could be observed, suggesting that this phenomenon may be restricted to male rats. The results found in the present study also show that the consequences of maternal separation are different in female Wistar rats and female ethanol-preferring AA rats. In female AA rats, both MS15 and MS360 resulted in a lower ethanol intake and preference compared to the AFR rats (Roman et al., 2005). Again, this is in contrast to the findings of similar effects after maternal separation in male ethanol-preferring AA rats (Roman et al., 2003) and in male Wistar rats (Ploj et al., 2003a). The mechanisms underlying sex differences in the consequences of maternal separation are unknown but may include differences in neuronal development, in mother–infant relations and in endocrine regulation. The ethanol intake may also be influenced by the estrous cycle (Ford et al., 2002; Roberts et al., 1998). The estrous cycle was not controlled in the present study but in a recent study using the same experimental protocol it was shown that the ethanol intake was similar throughout the estrous phases (Roman et al., 2005). Furthermore, since the statistical evaluation involved comparisons of mean ethanol intake during longer periods of time it is unlikely that a possible individual variation should influence the present data.

Previous studies have pointed at a connection between maternal separation responses and the endogenous opioid (Irazusta et al., 1999; Kalinichev et al., 2001; Ploj and Nylander, 2003; Ploj et al., 1999, 2001, 2003a,b) and N/OFQ systems (Ploj and Nylander, 2003; Ploj et al., 2001, 2002). The development of the opioid systems is not uniform and quite complex (McDowell and Kitchen, 1987) and postnatal environmental influences may interfere with the ontogeny of the opioid systems and thereby their function in adult life. Separation-induced effects on opioid peptide systems have been observed in brain areas related to the hypothalamic–pituitary–adrenal (HPA) axis in male Wistar rats (Ploj et al., 2002, 2003b). In the present study, only the hypothalamus was affected, with lower ir DYNB levels in animals exposed to MS15. In male rats, separation-induced changes also in ir MEAP and N/OFQ levels and in addition ir DYNB levels were altered in the neurointermediate pituitary lobe (Ploj et al., 2002, 2003b). Female rats thus seem less affected by early environmental manipulations in these areas and these results are in agreement with previous findings of a differential outcome of maternal separation in female (Ploj et al., 2001) and male (Ploj et al., 1999) Sprague–Dawley rats.

Ir peptide levels were altered in the nucleus accumbens, cortical areas and the amygdala, brain areas related to drug dependence mechanisms (Koob and Nestler, 1997). High levels of opioid peptides and receptors are present in, and involved in regulation of, brain areas related to dopaminergic functions (Christensson-Nylander et al., 1986; Steiner and Gerfen, 1998; You et al., 1994). The mesocorticolimbic reward system, for instance, originating in the ventral tegmental area and projecting via the nucleus accumbens to the frontal cortex, has been shown to be under tonic control by opposing opioid systems (Spanagel et al., 1992). In the present study, MS15 resulted in higher ir MEAP levels in the nucleus accumbens, whereas the other peptide levels were unaffected. In the medial prefrontal cortex, both MS15 and MS360 resulted in higher ir MEAP peptide levels. The length of separation thus had no effect on basal levels in this brain area, suggesting that the contact with the rats during the separation procedures (that was minimal in the AFR rats) were more likely to cause these changes. The same observation was found in the amygdala where both MS15 and MS360 induced a reduction in ir N/OFQ levels. Ir DYNB levels were not altered in any of these brain areas. Again, different results were obtained in the female rats compared to male rats. Ir DYNB and N/OFQ levels were

less affected in the female rats, whereas on the other hand, ir MEAP levels were altered in brain areas not affected in male rats (Ploj et al., 2002, 2003b). Further studies are now needed to understand these sex differences in neurochemical consequences of maternal separation and to elucidate whether they could contribute to the observed sex differences in voluntary ethanol intake.

In the present study we also examined whether the 4-week period of voluntary ethanol intake could affect peptide levels differently in MS15, MS360 and AFR rats, respectively. That is, could ethanol induce different effects depending on early environmental influence? Ethanol has a complex mechanism of action, affecting several transmitter systems, including the endogenous opioid system. Studies on the effects of ethanol exposure on endogenous opioid peptide systems have shown various results depending on brain area examined, dosage, and experimental conditions (Cowen and Lawrence, 2001; Gianoulakis, 2004; Herz, 1997; Oswald and Wand, 2004). Most studies have used experimenter-controlled administration and/or forced administration paradigms and few report effects of voluntary ethanol consumption (De Waele and Gianoulakis, 1994; Nylander et al., 1994). It is therefore interesting to note that in the present study, a general observation was that exposure to a free choice of ethanol and water in a non-preferring female Wistar rat, leading to a voluntary moderate ethanol intake, causes specific alterations in ir opioid and N/OFQ peptide levels in several brain areas (Table 2). In most brain areas studied, regardless of experimental group, lower ir DYNB and N/OFQ levels were detected in ethanol-drinking rats. Exceptions were the hippocampus and the pituitary lobe where higher ir DYNB levels were found, and the periaqueductal gray where higher ir N/OFQ levels were present after ethanol drinking. The overall effects of voluntary ethanol drinking on ir MEAP levels were opposite to those found for DYNB and N/OFQ, i.e. higher levels were detected in all areas except for the amygdala. Another interesting finding was that even though no statistical differences in ethanol intake between experimental groups were observed, it was shown that 4 weeks of voluntary ethanol intake affected MS15 and MS360 rats differently than AFR rats in several brain areas. In the hypothalamus, no significant differences in ir DYNB levels were observed between experimental groups after voluntary ethanol intake, i.e. the separation-induced differences were abolished. The opposite was observed for ir MEAP levels, maternal separation per se did not affect peptide levels but after voluntary ethanol drinking MS15 animals had higher levels than the other groups. Also in the frontal cortex and neurointermediate lobe of the pituitary gland, ethanol intake affected peptide levels differently, leading to differences in ir DYNB levels between experimental groups after the period with access to ethanol. In the medial prefrontal cortex and the nucleus accumbens, the observed separation-induced differences in ir MEAP levels between the experimental groups no longer remained after the period of voluntary ethanol intake. Finally, in the amygdala, separation-induced changes in ir N/OFQ levels were abolished after voluntary ethanol. Taken together, these results indicate that the early environmental setting may influence the response to adult use of ethanol, shown here as differential effects on opioid and N/OFQ levels in several brain areas.

In conclusion, voluntary ethanol intake in female Wistar rats was not altered by the maternal separation procedures, MS15 and MS360. Ir peptide levels were affected in the hypothalamus, medial prefrontal cortex, nucleus accumbens and the amygdala. The effects were different from those previously described in male Wistar rats and give further evidence for sex differences in the consequences of maternal separation. Furthermore, even though the ethanol drinking patterns were similar in the experimental groups, it was shown that a moderate voluntary ethanol drinking can induce different neurochemical effects dependent on prior early environmental setting, especially in areas related to the HPA axis and drug dependence mechanisms.

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