

Research report

The expression of opioid genes in non-classical reward areas depends on early life conditions and ethanol intake



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ARTICLE INFO

Article history:

Received 25 October 2016

Received in revised form 21 April 2017

Accepted 8 May 2017

Available online 13 May 2017

ABSTRACT

The young brain is highly sensitive to environmental influences that can cause long-term changes in neuronal function, possibly through altered gene expression. The endogenous opioid system continues to mature after birth and because of its involvement in reward, an inadequate maturation of this system could lead to enhanced susceptibility for alcohol use disorder. Recent studies show that the classical reward areas nucleus accumbens and ventral tegmental area are less affected by early life stress whereas endogenous opioids in non-classical areas, e.g. dorsal striatum and amygdala, are highly responsive. The aim was to investigate the interaction between early life conditions and adult voluntary ethanol intake on opioid gene expression. Male Wistar rats were exposed to conventional rearing, 15, or 360 min of daily maternal separation (MS) postnatal day 1–21, and randomly assigned to ethanol or water drinking postnatal week 10–16. Rats exposed to early life stress (MS360) had increased opioid receptor gene (*Oprm1*, *Oprd1* and *Oprk1*) expression in the dorsal striatum. Ethanol drinking was associated with lower striatal *Oprd1* and *Oprk1* expression solely in rats exposed to early life stress. Furthermore, rats exposed to early life stress had high inherent *Pomc* expression in the amygdala but low expression after ethanol intake. Thus, adverse events early in life induced changes in opioid gene expression and also influenced the central molecular response to ethanol intake. These long-term consequences of early life stress can contribute to the enhanced risk for excessive ethanol intake and alcohol use disorder seen after exposure to childhood adversity.

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1. Introduction

Brain development is associated with high sensitivity to environmental influences that can cause long-term changes in neuronal function through altered gene expression. The endogenous opioid system continues to mature after birth and abundant evidence indicates that endogenous opioids closely interact with the social environment early in life (Lutz and Kieffer, 2013a; Machin and Dunbar, 2011). In humans and non-human primates, a variant of the gene encoding the μ -receptor can influence attachment behaviour and the relationship between the parent and child (Barr et al., 2008; Copeland et al., 2011). In rodent models of parental neglect and loss, prolonged maternal separation induces long lasting alternations in the levels of endogenous opioids in areas related to reward and habitual drug consumption (Nylander and

Roman, 2012) as well as alters the adult response to opioid receptor antagonists (i.e. naltrexone) (Daoura and Nylander, 2011) and agonists (i.e. morphine) (Kalinichev et al., 2001). It is hypothesised that endogenous opioids are important to motivate attachment behaviour between parent and offspring possibly through the release of opioids that mediate reward during social contact (Machin and Dunbar, 2011; Nelson and Panksepp, 1998). Accordingly, Moles et al., 2004 showed that mice pups lacking the μ -receptor emitted fewer distress calls when separated from their mothers. These studies confirm important roles for opioids early in life and support the notion that the endogenous opioid system is targeted by early environmental influences.

Endogenous opioids are involved in a variety of physiological functions such as regulation of reward and mood (Le Merer et al., 2009; Lutz and Kieffer, 2013b; Yaksh and Wallace, 2011). In the reward pathways endogenous opioids contribute to the release of dopamine in the nucleus accumbens, partly through disinhibition of dopaminergic neurons in the ventral tegmental area (Spanagel and Weiss, 1999). However, reward and reinforcement are not solely mediated by these two classical reward areas but

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rather by several areas interacting in reward networks. In recent years, emerging evidence show that the dorsal part of striatum is implicated in reward and addiction, especially in the transition from voluntary to habitual drug intake (Everitt and Robbins, 2016). Further, it is hypothesised that both the amygdala and substantia nigra are necessary in the shift towards compulsory drug use (Belin et al., 2013). A common feature of these three structures, i.e. dorsal striatum, substantia nigra and amygdala, here denominated non-classical reward areas, is the rich content of opioid peptides and/or receptors (Le Merrer et al., 2009). Furthermore, it is demonstrated that the striato-nigral dopamine pathways are tightly regulated by opioid peptides (Christensson-Nylander et al., 1986; Herrera-Marschitz et al., 1986). Previous studies from our laboratory have shown that opioids in the nucleus accumbens and ventral tegmental area are mainly unaffected by early life stress. In contrast, opioid peptides in both the striatum and amygdala as well as voluntary ethanol consumption are highly affected by early life stress, and it has also been shown that ethanol induces different effects on opioids in the striatum and amygdala depending on early life environment (Gustafsson et al., 2008; Palm et al., 2013; Palm and Nylander, 2014).

The main hypothesis in the present study is that disturbance of social interactions early in life affects the development of the reward system and, in turn, contributes to higher susceptibility to develop addiction. Indeed, preclinical and clinical evidence point towards a relationship between exposure to early life stress or trauma and increased risk for subsequent alcohol- or substance use disorder (Enoch, 2011, 2012).

It is not yet known whether the long-term effects on peptides seen after early life stress is a result of altered gene expression or changes in enzymatic conversion of the peptides. The hypothesis in the present study is that early life stress causes alterations in expression levels. Therefore, the present study specifically aimed to investigate how early life stress, alone and in combination with subsequent ethanol intake, affects opioid receptor and prohormone gene expression in the dorsal striatum, substantia nigra and amygdala in outbred rats. A rodent model of early life stress (i.e. prolonged maternal separation) was used to evaluate the effects of different rearing conditions on the adult expression of genes coding for the μ receptor (*Oprm1*), the δ receptor (*Oprd1*) and the κ receptor (*Oprk1*) as well as proopiomelanocortin (*Pomc*), proenkephalin, (*Penk*) and prodynorphin (*Pdyn*). Furthermore, the expression levels were assessed in adult ethanol-drinking animals previously exposed to the different rearing conditions. Finally, the interaction effect between early life stress and ethanol were analyzed to assess whether early life stress alters the ethanol-induced effects on opioid expression.

2. Methods

2.1. Animals

All animal experiments were performed according to a protocol approved by the Uppsala ethics committee (C32/11) and in accor-

dance with the Swedish Legislation on Animal Experimentation (Animal Welfare Act SFS1998:56) and the European Communities Council Directive (86/609/EEC). Pregnant Wistar rats (RccHan: WI, gestation day 15, G15) were sourced from Harlan Laboratories B.V., Horst, The Netherlands. The dams were individually housed in standard macrolon cages (type IV; 59 × 38 × 20 cm) containing wood chip bedding and nesting material (40 × 60 cm; Cellstoff, Papyrus, Mölndal, Sweden) and kept under standard conditions (22 °C, 50 ± 10% humidity, 12 h light-dark cycle commencing at 06:00, ad libitum access to food pellets and tap water, masking background noise). For a graphic outline of the experiment see Fig. 1.

2.2. Maternal separation

Prolonged maternal separation was used to investigate the effects of early life stress. Maternal separation models are well established and are based on interference in the vital early interactions between the dam and the pup (Nylander and Roman, 2013; Pryce and Feldon, 2003). In the literature, several different control groups have been used when evaluating the effects of prolonged maternal separation (Nylander and Roman, 2013). Short-term maternal separation, here 15 min (MS15), is the optimal control to prolonged separations, here 360 min of maternal separation (MS360). The use of the MS15 group controls for possible daily handling effects and MS15 represents a more naturalistic environment; in the wild, the dam leaves the nest for shorter periods of time (Fleming and Rosenblatt, 1974; Grota and Ader, 1969). Animal facility reared (AFR) rats were included to evaluate expression levels in rats exposed to conventional handling during care taking. Since the aim was to investigate the long-term effects of early life stress the animals were group housed after weaning and left undisturbed until the commencement of adulthood.

The maternal separation procedure has previously been described in detail (Bendre et al., 2015; Comasco et al., 2015; Vrettou et al., 2015). Briefly, at birth (PND 0) the pups were sexed and cross fostered, each litter (6 males and 4 females) were randomly assigned into three parallel rearing conditions; 15 (MS15; $n = 7$ litters) or 360 min (MS360; $n = 8$ litters) of maternal separation or reared according to conventional animal facility conditions (AFR; $n = 7$). In the MS groups, the litters were separated from their dam for 15 or 360 min and the separations were performed every day from PND 1 to PND 21. In the AFR group, the animals were left undisturbed. One person performed the separations and care taking. On PND 22 all animals were weighed and weaned. Only male rats were used in further experiments since females are less sensitive towards the effect of maternal separation (Nylander and Roman, 2013). During adolescence the rats were housed in groups of three.

2.3. Voluntary ethanol drinking

To investigate how different rearing conditions affected the initial intake of alcohol the animals were single housed and exposed to an intermittent two-bottle free choice intake previously used to

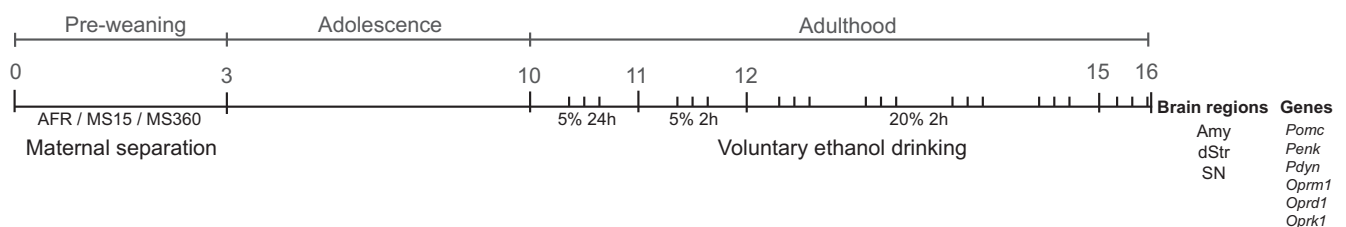


Fig. 1. Experimental outline. The numbers represent age in postnatal weeks. AFR, animal facility reared; MS, maternal separation 15/360 min, Amy, amygdala; dStr, dorsal striatum; SN, substantia nigra.

assess drinking pattern (Palm and Nylander, 2014). The drinking paradigm assesses sporadic voluntary intake and the neurobiology alterations that might occur prior dependence, withdrawal and habitual alcohol seeking. The limited access paradigm (2 h drinking sessions) was chosen to ensure less variation in biological parameters due to individual differences in drinking bouts in a 24 h access paradigm.

Prior to the ethanol drinking period started, the rats were individually housed and randomly assigned into drinking either water (AFR, $n = 9$, MS15, $n = 10$, MS360, $n = 10$) or ethanol (AFR, $n = 11$, MS15, $n = 10$, MS360, $n = 20$). For three consecutive days per week the rats had a free choice between water and ethanol for a total of six weeks. On the first week of drinking the rats were given access to 5% ethanol for 24 h, the second week the access was limited to 2 h and for the following four weeks the time of ethanol access was the same but the concentration of ethanol was increased to 20% (See Fig. 1). After the last 2 h session the animals were decapitated without prior anesthesia. For more detailed information see Bendre et al. (2015), Comasco et al. (2015) and Vrettou et al. (2015).

2.4. Tissue collection

The brains were immediately dissected with a pre-cooled rat brain matrix (ASI Instruments, Inc., Warren, NJ, USA). The brains were manually sliced with pre-cooled razor blades in coronal sections (1 mm slots) within 10–15 min. The brain regions were dissected on ice from the sections with the guidance of a rat brain atlas (Paxinos and Watson, 2007). The dorsal striatum (caudate putamen) was collected from sections between approximately fig. 15 (bregma 2.16 mm) and fig. 34 (bregma -0.12 mm) with the ventral striatum (nucleus accumbens) excluded, the amygdala between fig. 48 (bregma -1.80) and fig. 60 (bregma -3.24 mm) and the substantia nigra between fig. 71 (bregma -4.56) and fig. 84 (bregma -6.12). The tissue samples were immediately frozen on dry ice and stored at -80 °C until gene expression analysis.

2.5. Gene expression analyses

To purify and isolate RNA from dorsal striatum, substantia nigra and amygdala, All Prep DNA/RNA/miRNA Universal Kit (Qiagen®,

Sollentuna, Sweden) was used according to the manufacturer's instruction. Isolated RNA was quantified using a Nanodrop ND 1000 spectrophotometer. RNA was converted to cDNA by using the QuantiTect Reverse Transcription Kit (Qiagen AB, Sollentuna, Sweden) including a genomic wipeout reaction at 42° at 5 min. The synthesis of cDNA was performed a 42 °C for 30 min and inactivated at 95 °C. Newly synthesized cDNA was diluted 20 times and stored at -20 °C. The diluted cDNA was used to assess the expression of *Pomc*, *Penk*, *Pdyn*, *Oprm1*, *Oprd1* and *Oprk1* as well as house-keeping genes *Actb*, *Gapdh* and *Rpl19* using the CFX96 Touch Real-Time PCR detection System real time PCR. Primers were designed using Primer 3 (<http://frodo.wi.me.edu/>) and crosschecked using Primer Map (http://www.bioinformatics.org/sms2/primer_map.html) (Table 1). The final reaction mixture contained SYBR® Green Supermix (Bio-Rad Sweden), 0.4 μM of forward and reverse primer (Thermo Scientific, Germany), cDNA template and MQ-water. Each sample was run in triplicates. To control for gDNA contamination an on column DNase treatment was performed during extraction, prior cDNA synthesis a gDNA wipe-out extraction was utilized and the primers were designed across two adjacent exons to avoid unspecific amplification of gDNA. Moreover, each real time PCR plate contained positive internal controls.

2.6. Data analyses

Statistical analyses were performed using Statistica 12 (Statsoft) and SPSS version 22 (IBM). To analyse the effects of rearing as well as rearing and ethanol intake on gene expression the Kruskal Wallis test was used together with the Mann-Whitney *post hoc* test. The overall significant criterion was set to $p < 0.05$. To investigate possible interactions with a two-factor analysis (rearing and intake) the MS15 group was used in comparison with MS360. These groups have been exposed to similar handling during the first three weeks of life and therefore the duration of the separation is the only factor that differs between the groups and hence the AFR animals are excluded from this analysis. The two-factor analysis was tested using the Univariate General Linear Model (GLM) test, two-way ANOVA with type III sum of squares. Correlation analyses were performed using the non-parametric Spearman test. The correlation analyses were done to test possible associations in gene expression within and between the different brain regions

Table 1
Primers for gene expression assessment.

Gene	Gene Name	Accession Number	Product Size (bp)	Primer		Sequence	Location (bp)
				Tm (°C)	Ta (°C)		
Oprd1	Opioid receptor, Delta 1	NM_012617.1	124	85	64.5	F: 5'ggcaaggccaagctgatcaacat 3' R: 5'tggggaactggagcgtgcat 3'	592–716
Oprk1	Opioid receptor, Kappa 1	NM_017167.2	130	81.5	62.3	F: 5' ttggctactggcatcatctg 3' R: 5' gaacagggtcccaccaggaat 3'	616–746
Oprm1	Opioid receptor, Mu 1	NM_001304736.1	153	81.5	62.3	F: 5' ctccatgtgtcacagccatt 3' R: 5' ctgccagagcaaggttga 3'	407–559
Pdyn	Prodynorphin	NM_019374.3	157	87	62.3	F: 5' cctgtcctgtgttcctgt 3' R: 5' agaggcagtcagggtgagaa 3'	304–460
Penk	Proenkephalin	XM_006237835.1	134	87.5	63.5	F: 5' agactttgcatctggctgt 3' R: 5' agtgtgcatggcaggaagt 3'	196–330
Pomc	Proopiomelanocortin	NM_139326.2	223	89	60	F:5' caagaggagctggaaggcagc 3':5' tcaactggcccttctgtgc 3'	607–830
Actb	Actin, Beta	NM_031144.3	81	83	60	F:5'cactgccatctctctct3' R:5'aaccgctcattgccgatagt3'	762–842
Gapdh	Glyceraldehyde-3-Phosphate Dehydrogenase	NM_017008.4	90	84	60	F:5'acatgccgctggagaaacct3' R:5'gccagatgccctttagtgg3'	805–894
Rpl19	Ribosomal Protein L19	NM_031103.1	89	83	62	F:5'tcgccaatgccactctctgtc3' R:5'agccgggaatggacagtcac3'	123–211

Forward and reverse primers for gene expression assessment with respective amplicon size, melting temperature (°C), annealing temperature (°C) and location. Abbreviations; F: forward; R: reverse; Ta: annealing temperature; Tm: melting temperature

and also between gene expression and ethanol intake during the last session as well as the average consumption the last week. The ethanol intake during the last session is the intake parameter being closest to the neurobiological analysis, i.e. the ethanol consumed during the 2 h session preceding sacrifice. Since the ethanol intake during a single session can vary in a freely drinking paradigm, the average consumption the three sessions the last week was also used in the correlation analysis. Thus, the ethanol consumption during the last week represents the individual drinking pattern close to sacrifice and neurobiological analysis. Regarding the substantia nigra, no data were considered for the *Oprd1* because of the high number of samples ($n = 20$) with expression below detection. The expression levels of the other genes in substantia nigra were also low, although more randomly distributed between groups. Thus the interpretation of the results for this region should be cautious.

3. Results

3.1. Voluntary ethanol intake and body weight

The animals' drinking pattern and detailed information about ethanol intake have previously been described in detail (Bendre et al., 2015; Comasco et al., 2015; Vrettou et al., 2015). The time point for neurobiological analysis was chosen to coincide with the time point when the MS360 animals usually start to deviate from the other rats in terms of ethanol consumption (Daoura and Nylander, 2011) and group level statistics revealed that there were no differences in overall ethanol intake between the three different rearing conditions (Comasco et al., 2015). However, there was a significant difference in ethanol consumption over time; MS15 and AFR rats had a more stable intake pattern over time whereas the pattern in the MS360 rats were more diverse with a larger subpopulation of rats increasing intake over time and drinking >1.5 g/kg ethanol in a 2 h session (defined as high-drinking animals) towards the end of the experiment (Bendre et al., 2015; Comasco et al., 2015). The proportion of high-drinking animals were highest in the MS360 group (35%) compared to the MS15 (10%) and AFR (18%) groups (Comasco et al., 2015). The ethanol consumption patterns and the presence of "responders", i.e. rats that acquire a higher ethanol consumption after early life stress, and "non-responders", i.e. rats that seem largely unaffected by early life stress, were similar to previous studies using the same

maternal separation model (Nylander and Roman, 2013; Roman and Nylander, 2005).

The body weight has been reported previously (Todkar et al., 2015). The MS360 rats had slightly lower body weight at postnatal week 16. However, the body weight increase was similar between groups and body weight had no effect on gene expression when used as a covariate in the general linear model.

3.2. Do rearing conditions affect the expression of opioid related genes in adulthood?

To examine whether gene expression differ in adult animals previously subjected to different rearing conditions, the gene expression in water-drinking AFR, MS15 and MS360 rats were compared.

3.2.1. Effects of rearing conditions on gene expression in the dorsal striatum

In the dorsal striatum, the opioid receptor gene expression was dependent on rearing conditions (Table 2). Rats exposed to 360 min of maternal separation had a higher expression of *Oprd1* and *Oprk1* compared to MS15 and AFR rats (Fig. 2). The *Oprm1* expression was higher in the MS360 animals compared to AFR (Fig. 2). The correlation between opioid receptor genes in dorsal striatum differed in the experimental groups (Table 3); one positive correlation was found in the MS15 group (*Oprd1* and *Oprm1*), no correlations in the MS360 animals, whereas positive correlations were found in the expression of all three receptor genes in the AFR animals. The expression of the prohormones in the dorsal striatum was independent of rearing conditions (Table 2).

3.2.2. Effects of rearing conditions on gene expression in the amygdala

In the amygdala, the *Pomc* expression was dependent on rearing conditions (Table 2); MS360 rats displayed the highest level of expression and AFR rats the lowest, whereas the MS15 rats had an intermediate *Pomc* expression (Fig. 3). The rearing conditions alone did not affect any other gene analyzed in the amygdala (Table 2).

3.2.3. Effects of rearing conditions on gene expression in the substantia nigra

In the substantia nigra, there were no differences between the MS360, MS15 and AFR groups in neither receptor nor prohormone expression levels (Table 2).

Table 2
Effects of rearing conditions on gene expression in amygdala, dorsal striatum and substantia nigra.

Brain region	Gene	AFRw (n)	MS15w (n)	MS360w (n)	Kruskal-Wallis
Amygdala	<i>Oprd1</i>	9	10	10	H = 0.81; <i>p</i> = 0.67
	<i>Oprk1</i>	9	10	10	H = 0.77; <i>p</i> = 0.68
	<i>Oprm1</i>	9	10	10	H = 0.16; <i>p</i> = 0.92
	<i>Pdyn</i>	9	10	10	H = 0.77; <i>p</i> = 0.68
	<i>Penk</i>	9	10	10	H = 0.32; <i>p</i> = 0.85
	<i>Pomc</i>	8	10	10	H = 12.34; <i>p</i> = 0.0021
Dorsal striatum	<i>Oprd1</i>	9	9	10	H = 9.96; <i>p</i> = 0.0069
	<i>Oprk1</i>	9	10	10	H = 11.8; <i>p</i> = 0.0027
	<i>Oprm1</i>	9	10	10	H = 6.56; <i>p</i> = 0.038
	<i>Pdyn</i>	9	9	10	H = 0.99; <i>p</i> = 0.61
	<i>Penk</i>	9	10	10	H = 3.51; <i>p</i> = 0.17
	<i>Pomc</i>	8	9	9	H = 3.28; <i>p</i> = 0.19
Substantia nigra	<i>Oprd1</i>	n.d			
	<i>Oprk1</i>	7	9	8	H = 1.07; <i>p</i> = 0.58
	<i>Oprm1</i>	5	10	10	H = 0.39; <i>p</i> = 0.82
	<i>Pdyn</i>	7	8	10	H = 1.75; <i>p</i> = 0.42
	<i>Penk</i>	7	10	9	H = 0.58; <i>p</i> = 0.97
	<i>Pomc</i>	7	10	9	H = 0.31; <i>p</i> = 0.85

AFR, $n = 9$, MS15, $n = 10$, MS360, $n = 10$. AFR, animal facility rearing; MS, maternal separation 15/360 min; n.d, not detectable; *Oprd1*, opioid receptor, delta 1; *Oprk1*, opioid receptor, kappa 1; *Oprm1*, opioid receptor, mu 1; *Pdyn*, prodynorphin; *Penk*, proenkephalin; *Pomc*, proopiomelanocortin; w, water.

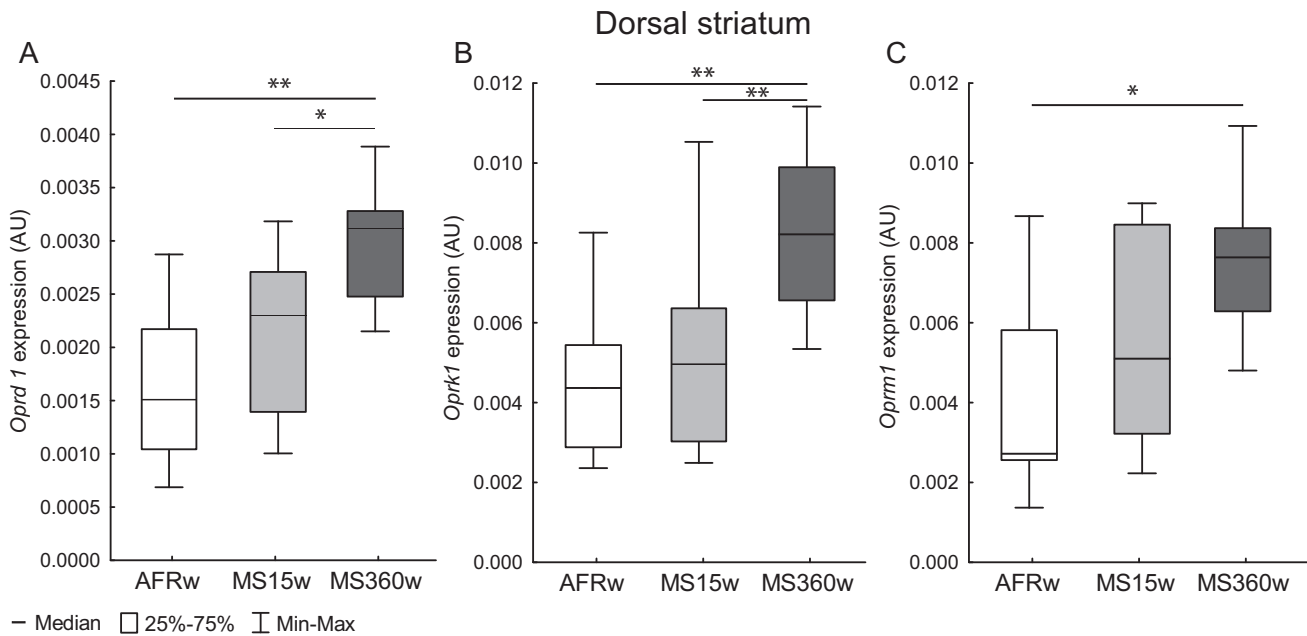


Fig. 2. Effects of rearing conditions on A) *Oprd1*, B) *Oprk1* and C) *Oprm1* gene expression in the dorsal striatum. *: $p \leq 0.05$; **: $p \leq 0.01$ (Mann-Whitney *U* test). AFR, animal facility rearing; AU, arbitrary units; MS, maternal separation 15/360 min; w, water drinking animals; *Oprd1*, opioid receptor delta 1; *Oprk1*, opioid receptor kappa 1; *Oprm1*, opioid receptor mu 1.

Table 3
Correlations between opioid receptor genes in the dorsal striatum.

Genes	AFRw	MS15 w	MS360 w	AFRe	MS15e	MS360e
<i>Oprd1</i> vs. <i>Oprk1</i>	0.82	0.62	0.61	0.76	0.73	0.78
<i>Oprd1</i> vs. <i>Oprm1</i>	0.87	0.80	0.14	0.88	0.84	0.73
<i>Oprk1</i> vs. <i>Oprm1</i>	0.92	0.61	0.21	0.76	0.90	0.68

The table shows the Spearman ρ value and bold numbers indicate a p -value < 0.01 . AFR, animal facility reared; e, ethanol; MS, maternal separation 15/360; *Oprd1*, opioid receptor, delta 1; *Oprk1*, opioid receptor, kappa 1; *Oprm1*, opioid receptor, mu 1; w, water.

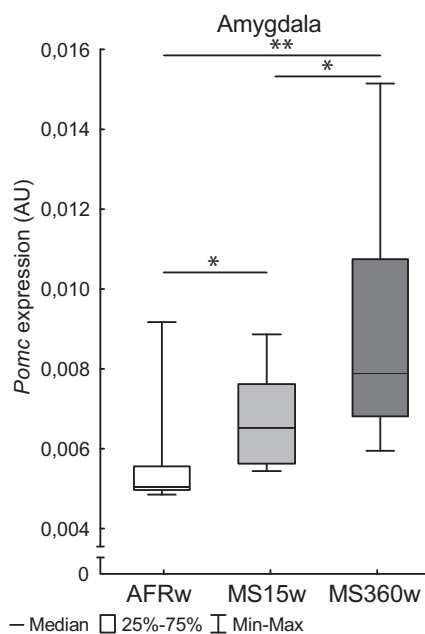


Fig. 3. Effects of rearing conditions on *Pomc* expression in the amygdala. *: $p \leq 0.05$; **: $p \leq 0.01$. (Mann-Whitney *U* test). AFR, animal facility rearing; AU, arbitrary units; MS, maternal separation 15/360 min; *Pomc*, proopiomelanocortin; w, water.

3.3. Does gene expression differ in ethanol-drinking animals exposed to different rearing conditions?

A comparison of the gene expression in the adult ethanol-drinking animals previously exposed to AFR, MS15 or MS360 revealed no difference between groups in any of the three brain regions (Table 4). Thus, the differences between groups seen in the inherent expression of opioid receptor genes in the dorsal striatum were no longer present in the ethanol-drinking rats. In addition and opposite to the findings in water drinking animals, correlation analysis revealed that ethanol-drinking maternally separated rats had positive correlations between expression levels of the opioid receptor genes similar to those seen in the AFR group (Table 3).

3.4. Does early life stress alter the response to ethanol drinking?

A two-factor analysis was used to investigate whether voluntary ethanol drinking in adulthood could induce different effects depending on early life stress. The main effect of intake (ethanol or water) and maternal separation (early life stress, MS360, or control, MS15) and the interaction between these factors were analyzed to investigate whether voluntary ethanol drinking in adulthood could induce different effects depending on early life stress.

Table 4
Effects of rearing conditions and voluntary ethanol drinking on gene expression.

Brain region	Gene	AFRe	MS15e	MS360e	Kruskal-Wallis
Amygdala	Oprd1	11	10	20	H = 1.33; p = 0.51
	Oprk1	11	10	20	H = 1.53; p = 0.46
	Oprm1	11	10	20	H = 0.11; p = 0.95
	Pdyn	11	10	20	H = 2.68; p = 0.26
	Penk	11	9	19	H = 1.72; p = 0.42
	Pomc	11	10	19	H = 2.55; p = 0.28
Dorsal striatum	Oprd1	11	10	20	H = 1.09; p = 0.58
	Oprk1	11	10	20	H = 2.04; p = 0.36
	Oprm1	11	10	20	H = 0.68; p = 0.71
	Pdyn	11	10	20	H = 1.99; p = 0.37
	Penk	11	10	20	H = 0.53; p = 0.77
	Pomc	11	10	20	H = 1.22; p = 0.54
Substantia nigra	Oprd1	n.d.			
	Oprk1	11	7	16	H = 0.058; p = 0.97
	Oprm1	11	5	17	H = 2.66; p = 0.26
	Pdyn	10	6	15	H = 4.17; p = 0.12
	Penk	11	7	16	H = 3.00; p = 0.22
	Pomc	10	8	17	H = 5.17; p = 0.076

AFR, n = 11, MS15, n = 10, MS360, n = 20. AFR, animal facility rearing; MS, maternal separation 15/360 min; n.d. not detectable: Oprd1, opioid receptor delta 1; Oprk1, opioid receptor kappa 1; Oprm1, opioid receptor mu 1; Pdyn, prodynorphin; Penk, proenkephalin; Pomc, proopiomelanocortin; w, water.

3.4.1. Ethanol-induced effects in the dorsal striatum

In the dorsal striatum, an interaction between rearing and intake was found in *Oprd1* ($F = 5.37$, $p = 0.025$, adjusted $R^2 = 0.25$) (Fig. 4A) and *Oprk1* expression ($F = 12.0$, $p = 0.001$, adjusted $R^2 = 0.34$) (Fig. 4B). In *Oprm1* no interaction was found ($F = 1.83$, $p = 0.18$, adjusted $R^2 = 0.02$). A main effect of ethanol intake was found with significant effects in *Oprd1* ($F = 10.3$, $p = 0.002$) and *Oprk1* ($F = 12.1$, $p = 0.001$) whereas no effect was present in *Oprm1* expression ($F = 1.28$, $p = 0.27$). The Mann-Whitney analysis revealed that only the ethanol-drinking MS360 animals differed

from their water-drinking counterparts in *Oprd1* ($U = 22$, $Z = -3.4$, $p < 0.001$) (Fig. 4A) and *Oprk1* ($U = 11$, $Z = -3.89$, $p < 0.001$) (Fig. 4B) expression. No main effect of rearing was found in the expression of the opioid receptors in the dorsal striatum (*Oprd1*, $F = 1.72$, $p = 0.20$; *Oprk1*, $F = 1.97$, $p = 0.17$; *Oprm1*, $F = 1.03$, $p = 0.32$).

3.4.2. Ethanol-induced effects in the amygdala

In the amygdala, an interaction between ethanol intake and rearing was found ($F = 5.11$, $p = 0.03$, adjusted $R^2 = 0.10$) in the

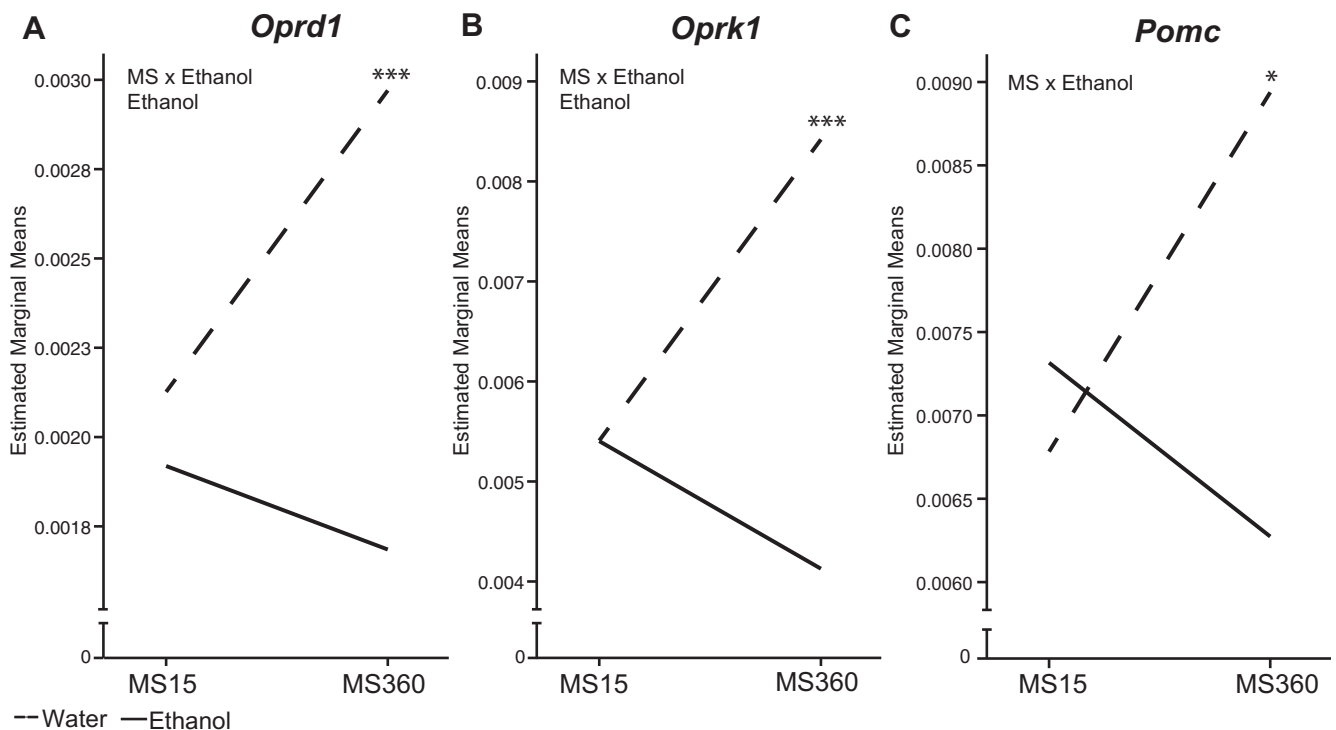


Fig. 4. Interaction and main effects between early life stress and adult ethanol drinking on A) *Oprd1* and B) *Oprk1* expression in the dorsal striatum and on C) *Pomc* expression in the amygdala. The Univariate General Linear Model test, two-way ANOVA with Type III sum of squares showed an interaction effect between rearing (maternal separation) and ethanol drinking (MS \times Ethanol) in the dorsal striatum and amygdala and a main effect of ethanol (Ethanol) in the dorsal striatum. * = $p < 0.05$, *** = $p < 0.001$ MS360 water vs. MS360 ethanol (Mann-Whitney U test). MS, maternal separation 15/360; Oprd1, opioid receptor delta 1; Oprk1, opioid receptor kappa 1; Pomc, proopiomelanocortin.

expression of *Pomc* (Fig. 4C). The Mann-Whitney test revealed a difference ($U = 45$, $Z = 2.27$, $p = 0.023$) between water and ethanol drinking MS360 rats whereas the expression was similar in the MS15 rats. No main effect of ethanol intake ($F = 2.27$, $p = 0.14$) or rearing ($F = 0.62$, $p = 0.44$) was found.

3.4.3. Ethanol-induced effects in the substantia nigra

No main effect of ethanol intake or rearing was observed and no interaction between ethanol intake and rearing was found in the substantia nigra.

3.5. Does ethanol intake correlate to gene expression?

Spearman correlations were used to assess the relationship between ethanol intake and gene expression. Correlations were tested in all groups and brain areas and the statistical significant results are shown below.

3.5.1. Correlations between ethanol intake and gene expression in the dorsal striatum

A negative correlation was found between the intake of ethanol on the last session and the dorsal striatal expression of *Oprd1* in the high drinking animals, i.e. rats drinking >1.5 g/kg/2 h ($r = -0.70$, $p = 0.05$).

3.5.2. Correlations between ethanol intake and gene expression in the amygdala

In the amygdala, a positive correlation was found between the average intake of the three last sessions and the expression of *Oprd1* when analyzing all animals ($r = 0.40$; $p = 0.01$), MS360 rats ($r = 0.56$; $p = 0.02$) and the animals with a high intake ($r = 0.74$; $p = 0.03$).

4. Discussion

The present study provides new and supportive evidence that early life environment interacts with the endogenous opioid system causing changes which may contribute to the previously observed differences in voluntary ethanol consumption and in ethanol-induced effects on opioids in adulthood. The endogenous opioid system consists of proopiomelanocortin, proenkephalin and prodynorphin, i.e. the prohormones that generate the endorphins, enkephalins and dynorphins, respectively, and the μ , δ and κ opioid receptors (Yaksh and Wallace, 2011). Our laboratory has previously published a series of experiments showing that maternal separation in combination with ethanol intake cause alterations in central levels of endogenous opioid peptides (Nylander and Roman, 2012). In these previous experiments we have identified that the endogenous opioids in especially the dorsal striatum and amygdala are sensitive towards the effects of early life stress and ethanol intake, while less long-term changes were seen in the classical reward areas (i.e. nucleus accumbens and ventral tegmental area). Here, we provide evidence for differences also in gene expression depending on exposure to early life stress and ethanol consumption in the amygdala and dorsal striatum. These results further strengthen the notion that habitual drinking involves endogenous opioids in these brain areas and that the ethanol-induced effects differ depending on early life history. The results are in line with several reports that reveal recruitment of other brain areas than the classical reward areas during the processes leading towards addiction and that differences in drug-induced effects in these areas may contribute to individual susceptibility to substance use disorders (Everitt and Robbins, 2016).

Amygdala, a brain area with important functions in the regulation of both stress and anxiety is also involved in modulating the

effects of alcohol (Gilpin et al., 2015; Koob, 2009). In the present study, animals exposed to early life stress i.e. 360 min of daily maternal separation had increased *Pomc* expression in the amygdala compared to both MS15 and AFR. Furthermore, voluntary drinking affected expression solely in the MS360 rats; the expression levels were lower than the water drinking MS360 rats and more close to the levels seen in MS15 rats. In the MS15 rats no difference was observed between ethanol- and water-drinking rats. These results indicate a normalizing effect, i.e. a reduction of the initial high inherent *Pomc* expression levels, after ethanol drinking in the MS360 rats. It was recently shown that *Pomc* expression is affected by MS360 in the hypothalamus (Todkar et al., 2015) where the main expression of *Pomc* occurs (Le Merrer et al., 2009). Here we report changes also in the amygdala where a minor fraction of the expression occurs (Civelli et al., 1982; Niikura et al., 2013). *Pomc* expresses a large prohormone, which in turn, is cleaved enzymatically into several biologically active peptides. It is therefore, within the framework of this study, difficult to interpret the outcome of the different ethanol-induced effects on specific peptides. Since beta-endorphin is one of the products from proopiomelanocortin, it is tempting to speculate that it might be a derangement of endogenous opioids underlying this difference. The literature regarding ethanol, beta-endorphins and amygdala is not conclusive but it has been shown that beta-endorphin increases in the central amygdala after acute exposure to ethanol (Lam and Gianoulakis, 2011; Lam et al., 2008). The difference in *Pomc* expression might also indicate increased presence of adrenocorticotrophic hormone (ACTH) and/or melanocyte-stimulating hormone (MSH). A functional study of Brunson et al., 2001 show that ACTH can act directly on neurons in the amygdala and down-regulate the expression of corticotrophin-releasing hormone, which is highly involved in the response to ethanol (Koob, 2008). Interestingly, ethanol exposure during adolescence and adulthood has been shown to reduce alpha-MSH in the central amygdala (Lerma-Cabrera et al., 2013; Navarro et al., 2008) an effect that resembles the interaction effect seen in the present study.

In the present study, voluntary intake of ethanol was positively correlated with the expression of *Oprd1* in the amygdala. This relationship was present when considering all animals, but more pronounced in the high-drinking individuals. Analysing the groups separately (i.e. MS360, MS15 and AFR), a positive correlation was only found in the MS360 group that also had the largest portion of high-drinking rats (Comasco et al., 2015). As mentioned above, the amygdala is known for regulating fear and anxiety and it is shown that stimulation of δ -receptors in the central nucleus of amygdala (CeA) reduces anxiety-like behaviours (Randall-Thompson et al., 2010). The anxiolytic effect of ethanol is well known and this effect is most likely due to GABAergic modulations in the amygdala (Gilpin et al., 2015). GABAergic neurons are the most abundant in the CeA (Swanson and Petrovich, 1998) and a great part of them are co-localized with enkephalin (Poulin et al., 2008; Veinante and Stoessel, 1997). Acute ethanol results in increased c-Fos activity in the CeA in both GABAergic and prepro-enkephalin expression neurons (Criado and Morales, 2000; Morales et al., 1998). Microinjection of opioid receptor antagonists into the CeA reduces self-administration of ethanol (Foster et al., 2004; Heyser et al., 1999) and electrophysiological experiments on δ -receptor knockout mice further confirm the relationship between ethanol, GABA and δ -receptors (Kang-Park et al., 2007). Together with these abovementioned studies, our results further confirm a relationship between ethanol intake and δ -receptor activity in the amygdala.

An interesting finding in the present study is the impact of early life stress on opioid gene expression in the dorsal striatum. Firstly, the expression of opioid receptors was dependent on rearing conditions. The MS360 rats had higher expression of *Oprd1* and *Oprk1*

in comparison to both MS15 and AFR rats, and higher *Oprm1* expression compared to AFR. These results confirm our previous findings that the outcome of being exposed to MS15 control and AFR conditions is more similar whereas the long-term effects of early life stress on opioid systems are evident in MS360 rats. Previous studies from our laboratory show that rats exposed to MS360 have low immunoreactive levels of Met-enkephalin-Arg⁶-Phe⁷ (MEAP), a marker of proenkephalin, in the dorsal striatum compared to MS15 (Gustafsson et al., 2008, 2007). Decreased levels of MEAP could be a cause or an effect of up-regulated δ -opioid receptors (i.e. increased expression of *Oprd1*), which corresponds well to the results in the present study.

Secondly, a main effect of ethanol and an interaction effect of MS and ethanol were seen in the expression of *Oprd1* and *Oprk1* in the dorsal striatum. The main effect of ethanol was mainly driven by the reduced expression in MS360 animals, since the water- and ethanol-drinking animals in the MS15 group were comparable to each other. These data are in line with previous findings showing that voluntary drinking resulted in higher striatal MEAP levels in MS360 rats whereas the MEAP levels were unaffected by ethanol in the MS15 animals (Gustafsson et al., 2007). The literature regarding pharmacological treatments with δ receptor antagonists and agonists and the effects on ethanol intake is inconclusive where studies have shown a decrease, increase or no effect on intake after δ receptor modulation (Ciccocioppo et al., 2002; Franck et al., 1998; Hyytia and Kiiänmaa, 2001; Ingman et al., 2003; Krishnan-Sarin et al., 1995; Margolis et al., 2008). Our results show that the expression of the *Oprd1* in MS360 animals with a history of early life stress respond differently to ethanol compared to MS15 rats. We have previously shown that the two groups respond differently to naltrrexone treatment (Daoura and Nylander, 2011). The lower expression of δ receptors seen in the present study and the previous findings of higher MEAP levels after ethanol could contribute to such differences in treatment response.

Thirdly, it is interesting that similar effects are seen in *Oprd1* and *Oprk1* expression in the dorsal striatum. Previous studies report effects on the κ opioid system after administration of several drugs of abuse (Butelman et al., 2012) but less is known about the impact of early life stress on these effects. A possible co-regulation of the δ and κ receptors in individuals exposed to early life stress and ethanol has to our knowledge not been reported but is of interest to be studied further. The exact role of opioid receptors in the dorsal striatum is not fully understood but they are regulators of transmission of other neurotransmitters such as dopamine, GABA and glutamate (Atwood et al., 2014; Banghart et al., 2015; Christensson-Nylander et al., 1986; Herrera-Marschitz et al., 1986; Steiner and Gerfen, 1998). Evidence that ethanol affects endogenous opioids in dorsal striatum can be seen in a series of experiment by Mendez and colleagues. They show that a single exposure of ethanol (2.5 g/kg) increased dorsal striatal binding of a δ receptor ligand after 2 h as well as increased the mRNA expression of *Penk* 1–2 h after the animals received the ethanol (Mendez et al., 2004, 2008). Furthermore, this effect in dorsal striatum was not found when using a μ receptor ligand in the binding assay (Mendez et al., 2003). In the studies of Mendez et al., acute and forced administration of ethanol was used whereas the present study employed a prolonged voluntary drinking model. Evidence from experiments involving voluntary drinking shows that high intake increases δ receptor activity and that intra-striatal injection of naltrindole (a selective δ receptor antagonist) decreased ethanol drinking (Nielsen et al., 2012). Taken together, depending on the route of administration (forced vs. voluntary drinking) and the length of the exposure (acute single vs. prolonged) the ethanol-induced effects on *Oprd1* expression differs.

4.1. Strengths and limitations

In the present study, a well-characterized model to study the impact of early life conditions was used with the inclusion of two different control groups (i.e. AFR and MS15) to increase comparisons to other studies using the same model. The AFR and MS15 displayed almost similar expression pattern and hence, confirms that it is the 360 min of maternal separation that causes the main changes in gene expression. As pointed out in the *Statistical analyses* paragraph the main advantage by using the MS15 group in the interaction test is the control for early handling, a factor that cannot be controlled for in the AFR group. Outbred rats were chosen to simulate the genetic diversion in the human population as well as to increase variety in alcohol drinking. Most of the studies covering ethanol-induced effects on endogenous opioids use forced exposure models and less is known about the effects of voluntary drinking. The voluntary alcohol paradigm was used to simulate episodic drinking reminiscent of habitual drinking patterns and to be able to pinpoint individuals susceptible for escalating intake. Based on previous studies on the impact of prolonged maternal separation on ethanol consumption (Nylander and Roman, 2013; Palm et al., 2013), the duration of the ethanol-drinking period was chosen to investigate the initial effects of ethanol drinking. Previous studies from our laboratory have shown that voluntary drinking alters immunoreactive levels of endogenous opioids and that these effects are dependent on supplier, early life environment and housing (Palm et al., 2013, 2012; Palm and Nylander, 2014). A main limitation of this study is the low anatomical resolution of the gene expression analysis; for example the dissection of the amygdala did not allow analysis of specific sub regions such as central and basolateral amygdala. Since only gene expression was investigated the consequences on the outcome on protein levels and functions can only be hypothesised. However, together with the previous findings from our research group regarding opioid peptide levels after maternal separation (Nylander and Roman, 2012), these results provide further evidence that early life stress affects the endogenous opioid system.

5. Conclusion

The present study provides further evidence that the endogenous opioid system in the dorsal striatum and amygdala is an important target for early life stress. Early life stress (i.e. 360 min of daily maternal separation) affects the pattern of opioid gene expression in these areas compared to control rats (i.e. 15 min of maternal separation) as well as conventionally reared rats. Interestingly, the response to ethanol was also altered in animals subjected to early life stress as evidenced by an interaction between voluntary ethanol drinking in adulthood and the expression of *Oprd1* and *Oprk1* in the dorsal striatum as well as *Pomc* in the amygdala. These long-term consequences of early life stress can contribute to the enhanced risk for excessive ethanol intake and alcohol use disorder seen after exposure to childhood adversity.

Authors' contribution

IN, LG: animal experiment design; LG: performing animal experiment; AT, SB: gene expression analyses; LG, EC: statistical analyses; LG: writing the first draft; IN, EC: critical revision of the manuscript; all authors: finalization and approval of the content of the manuscript.

Conflict of interest

None.

Acknowledgments

The authors wish to thank Maria Vrettou, Megha Bendre, and Wout Boon at the Dept. of Neuroscience, Neuropsychopharmacology group and Lova Segerström and Marita Berg at the Dept. of Pharmaceutical biosciences, Neuropharmacology, Addiction and Behaviour group, for their contribution to the laboratory work. The study was partially supported by funds from the Alcohol Research Council of the Swedish Alcohol Retailing Monopoly (2014-0040) and the Swedish Research Council (K2012-61X-22090-01-3) to I.N.; from the Fredrik and Ingrid Thuring foundation (2012, 2013, 2014), Lars Hierta's Minne foundation (2013), Swedish Brain Foundation (PS2013-0052), Lundberg's and Karlsson's foundation (2013), and Ankarstrand's foundation (2015) to E.C.; from the , the Swedish Alcohol Monopoly Research Council, the Swedish Council for Working Life and Social Research (FAS/FORTE), the Uppsala and Örebro Regional Research Council, the Fredrik and Ingrid Thuring's Foundation, the County Council of Västmanland, the Köni g-Söderströmska Foundation, and the Svenska Spel Research Foundation to K.W.N.; and from the National Board of Health and Welfare to H.H. (The RESUMÉ data collection). E.C. is a Marie Skłodowska Curie fellow and received funds from the Swedish Research Council (VR: 2015-00495) and EU FP7-People-Cofund (INCA 600398). The funding body had no role in the design of the study, collection and analysis of data and decision to publish.

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