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Research Report

Effects of prenatal stress and monoaminergic perturbations on the expression of serotonin 5-HT$_4$ and adrenergic β_2 receptors in the embryonic mouse telencephalon

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ABSTRACT

The serotonin 5-HT$_4$ receptor (5-HT$_4$R) is coded by a complex gene that produces four mRNA splice variants in mice (5-HT$_{4(a)}$R, 5-HT$_{4(b)}$R, 5-HT$_{4(e)}$R, 5-HT$_{4(f)}$R). This receptor has highly dynamic expression in brain development and its splice variants differ in their developmental trajectories. Since 5-HT$_4$Rs are important in forebrain function (including forebrain control of serotonergic activity in the brainstem), we investigated the susceptibility of 5-HT$_4$R expression in the mouse embryonic telencephalon to prenatal maternal stress and altered serotonin (5-hydroxytryptamine, 5-HT) levels. Because the gene coding the adrenergic β_2 receptor (β_2AR) is embedded in the 5-HT$_4$R gene, we also investigated whether 5-HT$_4$R mRNA levels were modulated by selective β_2AR agents. Timed-pregnant C57BL/6 mice were treated beginning at embryonic day (E) 14 and quantitative reverse-transcription polymerase chain reaction (qRT-PCR) was used to assess the mRNA levels of all 5-HT$_4$R splice variants and β_2AR in the embryonic telencephalon at E17. Maternal prenatal stress and 5-HT depletion with *p*CPA, a tryptophan hydroxylase inhibitor, reduced the levels of the 5-HT$_{4(b)}$R splice variant. Terbutaline (a selective β_2AR agonist) and ICI 118,551 (a selective β_2AR antagonist) had no effect on β_2AR and 5-HT$_4$R mRNA levels. These results show that prenatal stress and reduced 5-HT levels can alter 5-HT$_4$R expression in the developing forebrain and that some 5-HT$_4$R splice variants may be more susceptible than others.

1. Introduction

The serotonin (5-hydroxytryptamine, 5-HT) 5-HT$_4$ receptor (5-HT$_4$R) is coded by a complex gene that generates at least ten mRNA splice variants in humans and pigs and four splice variants in mice (5-HT$_{4(a)}$R, 5-HT$_{4(b)}$R, 5-HT$_{4(e)}$R, 5-HT$_{4(f)}$R) (Bockaert et al., 2004, 2006; Claeysen et al., 1999; Ray et al., 2009). The expression of this receptor in mouse and human brain development is highly dynamic (Lambe et al., 2011; Manzke et al., 2008; Slaten et al., 2010; Waeber et al., 1994, 1996) and, at least in the mouse brain, the 5-HT$_4$R splice variants have different developmental trajectories (Hernandez and Janušonis, 2010). This variant-specificity may carry important temporal and spatial information because 5-HT$_4$R splice variants differ in their constitutive activity (Claeysen et al., 1999, 2001), internalization properties (Mnie-Filali et al., 2010),

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and association with intracellular proteins (Joubert et al., 2004).

Altered 5-HT_4R function has been associated with autism spectrum disorders (ASDs) (Vincent et al., 2009), major depressive disorder (Lucas et al., 2007; Rosel et al., 2004), bipolar disorder (Hayden and Nurnberger, 2006), and attention deficit/hyperactivity disorder (Li et al., 2006). A recent study has suggested that 5-HT_4R is a molecular network hub that controls multiple neural processes (Hu et al., 2011), including those important for the neurobiology of several brain disorders. Different levels of 5-HT_4R expression have been reported in the amygdala of males and females, which may contribute to sex differences in the prevalence of affective disorders (Madsen et al., 2011).

Evidence suggests that 5-HT_4R may be a key component in the forebrain control of the brain serotonergic system. Some of this control is mediated by a projection that originates in the medial prefrontal cortex (mPFC) and terminates in the dorsal raphe nucleus (Gabbott et al., 2005; Goncalves et al., 2009; Hajos et al., 1998; Vertes, 2004). In the adult brain, cortical 5-HT_4Rs modulate the activity of this projection (Bockaert et al., 2011; Lucas et al., 2005) and can alter brain serotonin (5-hydroxytryptamine, 5-HT) levels by affecting raphe serotonergic neurons directly or through GABAergic interneurons (Celada et al., 2001; Jankowski and Sesack, 2004). It has implications for a number of brain disorders, including the biological action of antidepressants (Lucas et al., 2010; Vidal et al., 2009). Since in the embryonic mouse brain young mPFC neurons express 5-HT_4Rs before they establish synapses with raphe neurons (Slaten et al., 2010), a transient change in 5-HT_4R expression in the fetal mPFC can potentially result in permanent dysregulation of 5-HT levels in many brain regions.

In the present study, we investigated how maternal prenatal stress and 5-HT depletion affect the mRNA levels of all 5-HT_4R splice variants in the embryonic mouse telencephalon. Previous studies have shown that maternal stress and serotonergic perturbations can cause various alterations in the developing brain (Altamura et al., 2007; Miyagawa et al., 2011; Peters, 1990; Vitalis et al., 2007). We also investigated whether maternal stress and other environmental signals can affect the 5-HT_4R expression in the embryonic telencephalon through adrenergic β_2 receptors (β_2ARs). Evidence suggests that 5-HT_4Rs may be functionally associated with β_2ARs, since the β_2AR gene is nested within the 5-HT_4R gene (Bockaert et al., 2004) and 5-HT_4Rs can form heterodimers with β_2ARs (Berthouze et al., 2005, 2007). Also, concerns have been raised regarding a possible association between terbutaline (a selective β_2AR agonist used to treat preterm labor) and ASDs (Witter et al., 2009), in which serotonergic abnormalities have long been noted (Anderson, 2002).

2. Results

2.1. *Effects of prenatal stress, β_2AR agents, and 5-HT depletion on β_2AR mRNA levels*

Since the transcription of the 5-HT_4R gene can potentially be affected by the transcription of the β_2AR gene due to the nested arrangement of the genes (Bockaert et al., 2004; Gibson et al., 2005), we first investigated whether the β_2AR mRNA amount in the embryonic telencephalon was altered by any of the treatments used in the study (maternal prenatal stress, selective β_2AR agents (terbutaline and ICI 118,551), and 5-HT depletion with *p*CPA) (Fig. 1). The initial statistical model included these conditions as the fixed effect (Treatment). Adding a random litter effect to the intercept significantly improved the model ($L=16.8$, $p<0.0001$). Allowing each condition to have a different variance did not further improve the model ($L=4.60$, $df=4$, $p>0.3$). The final validated model revealed no significant Treatment effect ($F_{4,17}=0.65$, $p=0.63$). In summary, the analysis indicated the presence of inter-litter variability in the baseline β_2AR mRNA levels (with an estimated standard deviation of 17% of the baseline), but these levels were not significantly altered by any of the used experimental perturbations.

2.2. *Effects of prenatal stress and β_2AR agents on 5-HT_4R splice variants*

We next examined whether maternal restraint stress and a selective β_2AR agonist (terbutaline) affected the mRNA levels of the 5-HT_4R splice variants in the embryonic telencephalon. We also investigated whether a selective β_2AR antagonist (ICI 118,551), administered prior to restraint stress, attenuated the stress effect on the 5-HT_4R mRNA levels (Fig. 2). The selective β_2AR agents were included because stress-related signals can act on β_2ARs (Qu et al., 2008), β_2ARs have been shown to form heterodimers with 5-HT_4Rs (Berthouze et al., 2005, 2007), and the activity of one receptor in a heterodimer can affect the internalization and trafficing of the other receptor, with potential changes in its transcription (Renner et al., in press; Rozenfeld and Devi, 2011).

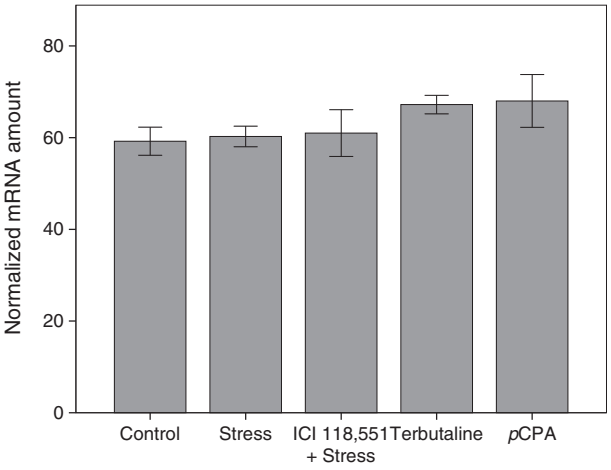

Fig. 1 – The normalized β_2AR mRNA amounts in the telencephalon of embryos (E17) from control dams injected with saline (Control) and from dams *(i)* injected with saline and exposed to maternal prenatal stress (Stress), *(ii)* injected with a β_2AR antagonist and exposed to maternal prenatal stress (ICI 118,551 + Stress), *(iii)* injected with a β_2AR agonist (Terbutaline), and *(iv)* injected with a tryptophan hydroxylase inhibitor (*p*CPA). The error bars are the standard errors of the means.

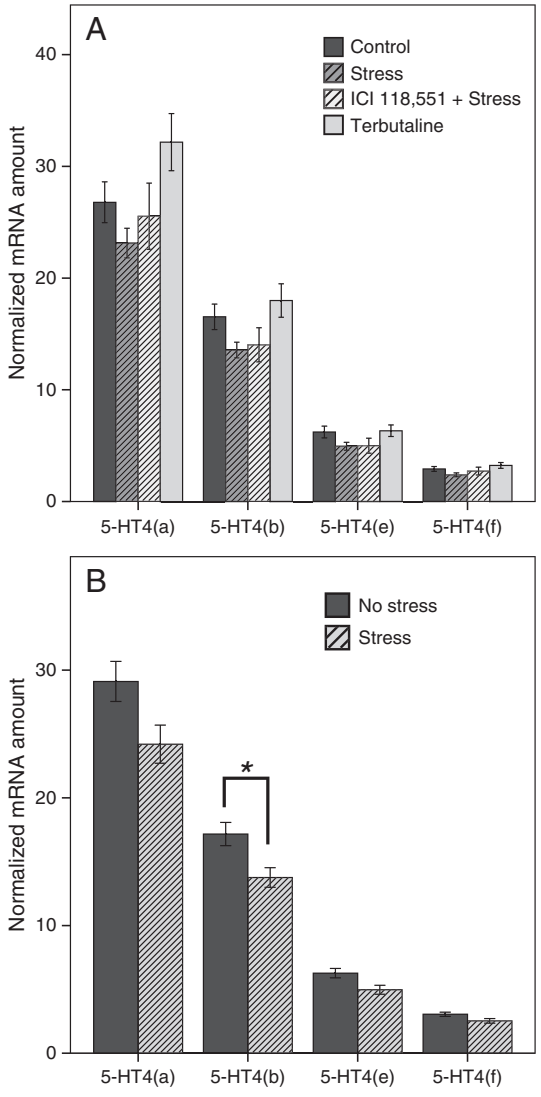

Fig. 2 – A: The normalized mRNA amounts of the four 5-HT$_4$R splice variants in the telencephalon of embryos (E17) from control dams injected with saline (Control) and from dams (i) injected with saline and exposed to maternal prenatal stress (Stress), (ii) injected with a β_2AR antagonist and exposed to maternal prenatal stress (ICI 118,551 + Stress), and (iii) injected with a β_2AR agonist (Terbutaline). B: The data obtained by pooling the two treatments with no stress (Control and Terbutaline) and the two treatments with stress (Stress and ICI 118,551 + Stress). The error bars are the standard errors of the means; $^*p<0.012$ (after the Bonferroni correction for the four comparisons (0.05/4)).

The initial statistical model (with Variant, Treatment, and Variant × Treatment as the fixed effects) was significantly improved by allowing each splice variant to have a different variance ($L=215.8$, $df=3$, $p<0.0001$) and was further improved by adding correlations (compound symmetry) among all splice variants ($L=87.7$, $df=1$, $p<0.0001$). No further improvement was obtained by adding a random litter effect to the intercept ($L=10^{-7}$, $p>0.4$), suggesting no variability among the litters. The final validated model showed no significant Variant × Treatment interaction ($F_{9,212}=1.60$, $p=0.12$) and no Treatment effect in the absence of the interaction term ($F_{3,221}=0.34$, $p=0.79$).

Since similar differences were observed between stressed and unstressed embryos irrespective of whether or not they had been treated with the selective β_2AR agents (Fig. 2A), we next combined these data into two groups disregarding the β_2AR treatment (Fig. 2B). This approach was further supported by the finding that neither the β_2AR agonist nor the β_2AR antagonist had a significant effect on the β_2AR mRNA levels (Fig. 1). Also, the correlations between the β_2AR mRNA levels and the mRNA levels of each 5-HT$_4$R splice variant were not significantly different from zero, in contrast to the strong correlations among the 5-HT$_4$R splice variants (Table 1). The two pooled conditions (no stress and stress) produced the same structure of the statistical model (the initial model: Variant, Treatment, Variant × Treatment; different variances for each splice variant: $L=229.5$, $df=3$, $p<0.0001$; correlations between the variants: $L=90.9$, $df=1$, $p<0.0001$; a random litter effect added to the intercept: $L=10^{-7}$, $p>0.4$). In the final validated model, the Variant × Treatment interaction was significant ($F_{3,220}=2.80$, $p=0.04$). There was no Treatment effect in the absence of the interaction ($F_{1,223}=0.57$, $p=0.45$). Consistent with this finding, only the decrease in the 5-HT$_{4(b)}$R levels was significant after the Bonferroni correction for the four comparisons (5-HT$_{4(a)}$R: $t_{55}=2.25$, $p=0.028$; 5-HT$_{4(b)}$R: $t_{55}=2.83$, $p=0.007$; 5-HT$_{4(e)}$R: $t_{55}=2.55$, $p=0.014$; 5-HT$_{4(f)}$R: $t_{55}=2.09$, $p=0.041$). The decrease in 5-HT$_{4(b)}$R mRNA was 20%.

2.3. *Effects of 5-HT depletion on 5-HT$_4$R splice variants*

We next assessed the susceptibility of all 5-HT$_4$R splice variants to low 5-HT levels in the developing brain. Depletion of 5-HT with *p*CPA strongly reduced the mRNA amount of the 5-HT$_{4(b)}$R splice variant in the embryonic telencephalon, with virtually no effect on the other three 5-HT$_4$R splice variants (Fig. 3).

The initial statistical model (with Variant, Treatment, and Variant × Treatment as the fixed effects) was significantly improved by allowing each splice variant to have a different variance ($L=86.7$, $df=3$, $p<0.0001$). Adding correlations between all splice variants further improved the model ($L=27.1$, $df=1$, $p<0.0001$). Adding a random litter effect to the intercept

Table 1 – The correlations between the mRNA amounts of the 5-HT$_4$R splice variants and β_2AR at E17 (since correlation measures only association strength, all experimental conditions were used). In each cell, the top number is the Pearson correlation (65 cases) and the bottom number is the *p*-value. By taking into account the Bonferroni correction for the ten cross-correlations, the significance level was set at 0.005 (*).

	5-HT$_4$R(b)	5-HT$_4$R(e)	5-HT$_4$R(f)	β_2-AR
5-HT$_4$R(a)	0.579* ($p<10^{-6}$)	0.707* ($p<10^{-10}$)	0.756* ($p<10^{-12}$)	0.059 ($p=0.64$)
5-HT$_4$R(b)		0.478* ($p<10^{-4}$)	0.472* ($p<10^{-4}$)	0.061 ($p=0.63$)
5-HT$_4$R(e)			0.576* ($p<10^{-6}$)	0.066 ($p=0.60$)
5-HT$_4$R(f)				0.067 ($p=0.59$)

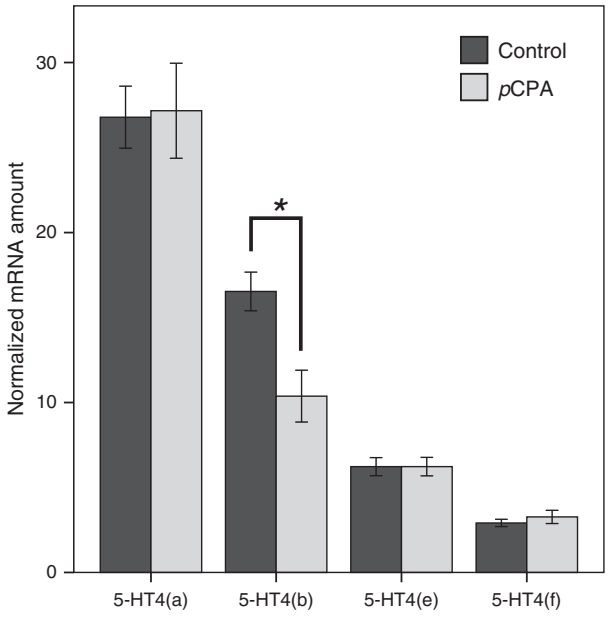

Fig. 3 – The normalized mRNA amounts of the 5-HT$_4$R splice variants in the telencephalon of embryos (E17) from control dams and from dams treated with *p*CPA. The error bars are the standard errors of the means; $^*p<0.012$ (after the Bonferroni correction for the four comparisons (0.05/4)).

did not further improve the model ($L=0.023$, $p>0.4$), suggesting no significant variability among the litters. The Variant × Treatment interaction in the final validated model was highly significant ($F_{3,92}=4.65$, $p=0.0045$). The decrease in 5-HT$_{4(b)}$R mRNA was 37% and significant after the Bonferroni correction for the four comparisons ($t_{23}=3.14$, $p=0.005$). The Variant × Treatment interaction remained significant if the data were analyzed with a mixed-design ANOVA with the Greenhouse–Geisser correction (Field, 2009), to account for the violation of the sphericity assumption ($F_{1.8,41.4}=3.72$, $p=0.037$).

3. Discussion

The obtained results demonstrate that prenatal stress and reduced 5-HT levels can alter 5-HT$_4$R expression in the developing telencephalon and that some 5-HT$_4$R splice variants may be more susceptible than others. They also suggest that these effects are not mediated or modulated by β_2AR receptors.

Binding studies in the adult brain have found that altered 5-HT levels typically cause compensatory changes in the availability of 5-HT$_4$Rs to ligands. On the one hand, an increase in 5-HT$_4$R binding has been observed in the brains of adult rats and young pigs following 5-HT depletion with *p*CPA (Ettrup et al., 2011; Licht et al., 2009) and in mice overexpressing the serotonin transporter (5-HTT) (Jennings et al., in press). On the other hand, a decrease in 5-HT$_4$R binding has been observed in the adult rat brain following chronic treatment with the selective serotonin reuptake inhibitors fluoxetine and paroxetine (Licht et al., 2009; Vidal et al., 2009) and in knockout mice lacking 5-HTT (Jennings et al., in press). Prenatal stress can increase 5-HT levels in the embryonic rat brain (Peters, 1990) and it may also increase the number of 5-HT immunoreactive neurons in the adult brains of prenatally exposed mice (Miyagawa et al., 2011). Taken together, these results appear to indicate that prenatal stress is likely to decrease 5-HT$_4$R expression in the embryonic telencephalon and that 5-HT depletion is likely to increase it.

Our results are not consistent with these predictions and suggest a more complex developmental mechanism. Both maternal stress and 5-HT depletion caused a decrease in 5-HT$_4$R mRNA levels, even though the strength of the effect varied among the conditions and splice variants. These shifts are remarkably similar to the results obtained in another study that has investigated serotonin 5-HT$_{1A}$ receptors (5-HT$_{1A}$Rs) in the developing rat brain (Lauder et al., 2000). In this study, prenatal exposure to 5-methoxytryptamine (5-MT), a non-specific 5-HT receptor agonist, has caused a moderate (but significant) decrease in 5-HT$_{1A}$R mRNA levels and prenatal depletion of 5-HT with *p*CPA has reduced 5-HT$_{1A}$R mRNA levels by nearly 50%. These changes are comparable to our findings not only in their direction, but also in their relative magnitudes (Figs. 2B and 3). Taken together, these findings suggest that, in development, the expression of at least some 5-HT receptors requires an optimal 5-HT level and that a deviation from this optimum may result in a lower number of mRNA transcripts. Abnormally low or high 5-HT levels may induce this change through the same mechanism or two different mechanisms. For example, low 5-HT levels might result in a failure to developmentally upregulate mRNA levels, perhaps in a splice-specific manner (Hernandez and Janušonis, 2010), whereas high 5-HT levels might lead to compensatory downregulation of mRNA levels. Generally, quantitative relationships between mRNA transcripts and receptor availability can be complex or counterintuitive due to the dynamics of the system (Janušonis, 2012). In this regard, several technical considerations are important for the interpretation of our and other related studies.

Most published studies have examined 5-HT$_4$R binding, whereas receptor mRNA levels were analyzed in our study and in Lauder et al. (2000). The measures obtained by these two technical approaches are not directly comparable. First, 5-HT$_4$R binding can change due to receptor internalization and recycling (Mnie-Filali et al., 2010), with no change in mRNA levels. Second, mRNA analysis cannot detect 5-HT$_4$Rs on the terminals of long-range projections that originate outside the telencephalon. There is strong evidence that 5-HT$_4$Rs can be expressed not only postsynaptically, but also presynaptically in distal axonal segments (Slaten et al., 2010; Vilaro et al., 2005). Third, binding studies cannot distinguish among 5-HT$_4$R splice variants; therefore, changes affecting only some of the variants can be masked by other, abundantly expressed variants.

A recent study has shown that early forebrain development depends on maternal 5-HT signals that reach the embryo through the placenta (Bonnin et al., 2011). While no information is currently available about how placental 5-HT synthesis is affected by maternal stress, *p*CPA is known to inhibit placental 5-HT synthesis (Bonnin et al., 2011). We cannot rule out that placental 5-HT might have contributed to

our findings, but we note that all experimental treatments began at E14, around the time when the mouse forebrain begins to switch to its endogenous 5-HT source, brainstem serotonergic afferents (Bonnin and Levitt, 2011).

The relatively small effect of maternal stress on 5-HT_4R levels may be due to the restraint length (one hour per day), which was low in comparison to that used in other reports. Daily restraint stress for a total of 1.5–3.0 h is considered standard or "weak" (Darnaudery et al., 2004; Holson et al., 1995; Miyagawa et al., 2011; Ribes et al., 2010; Vallee et al., 1997), and some researchers have used daily six-hour exposures to produce "strong" prenatal stress (Miyagawa et al., 2011). However, such long exposures increase the contribution of various confounding factors, such as muscle fatigue, thermoregulation, blood circulation, and food intake, and may consequently reduce the relevance of the findings to human research. Achieving an optimal balance between experimental effects in rodents and relevance for human research will require a better understanding of the dose-dependent effects of maternal prenatal stress (Mychasiuk et al., 2011).

The susceptibility of the 5-$HT_{4(b)}$R splice variant to altered 5-HT levels is interesting because in the adult brain this variant is readily internalized and recycled (Mnie-Filali et al., 2010). Consequently, it is well-positioned to rapidly respond to fluctuations of 5-HT levels at the protein level and may depend on a relatively stable mRNA pool. If the reduced availability of 5-$HT_{4(b)}$R mRNA persists into adulthood, it may have implications for brain disorders associated with dysfunction of 5-HT homeostasis.

4. Experimental procedures

4.1. *Animals*

Timed-pregnant C57BL/6 mice were purchased from Charles River Laboratories and housed individually on a 12:12 light–dark cycle (lights on at 07:00, off at 19:00) with free access to water and food. All procedures have been approved by the UCSB Institutional Animal Care and Use Committee.

4.2. *Treatment groups*

Dams were randomly assigned to one of five treatment groups. In the first (control) group, dams were given an intraperitoneal (i.p.) injection of saline once every day beginning at embryonic day (E) 14. In the second group, embryonic 5-HT was depleted by treating dams with DL-*para*-chlorophenylalanine methyl ester hydrochloride (*p*CPA; Sigma-Aldrich #C3635), a tryptophan hydroxylase inhibitor, dissolved in saline. Based on published reports (Vataeva et al., 2008; Vitalis et al., 2007), dams were given an injection of *p*CPA (200 mg/kg, i.p.) once every day beginning at E14. In the third group, dams were given an intraperitoneal injection of saline and immediately exposed to restraint stress by placing them in a 50 mL polystyrene centrifuge tube with air-holes for 1 h every day beginning at E14. Restraint stress has been used as a controlled stressor in many published studies (Darnaudery et al., 2004; Holson et al., 1995; Jones et al., 2010; Ribes et al., 2010; Vallee et al., 1997). In the fourth group, dams were exposed to restraint stress as dams in the third group, but instead of saline they were given an injection of ICI 118,551, a selective adrenergic β_2 antagonist (Sigma-Aldrich #I127), dissolved in saline. The dose of ICI 118,551 (2 mg/kg, i.p.) was based on published studies (Yalcin et al., 2010; Yu et al., 2010). In the fifth group, dams were treated with terbutaline hemisulfate, a selective adrenergic β_2 agonist (Sigma-Aldrich #T2528), dissolved in saline. Based on published reports (Thaker et al., 2006), dams were given an injection of terbutaline (5 mg/kg, i.p.) every day beginning at E14. Both terbutaline and ICI 118,551 could reach the embryonic brain because the mouse blood–brain barrier matures after birth (Daneman et al., 2010) and it is permeable to terbutaline and ICI 118,551 in adulthood (Hsu et al., 1994; Moresco et al., 2000). In all groups, the injected volume was kept constant (0.5 cm^3).

4.3. *Tissue collection and RNA isolation*

At E17, dams were terminally anesthetized with a mixture of ketamine (200 mg/kg) and xylazine (20 mg/kg), and their uterus was dissected and kept in 0.1 M phosphate-buffered saline (PBS, pH 7.4) on ice. Embryos were removed from the uterus, decapitated, and their brains were dissected with a fine forceps under a stereoscope. The telencephalon was isolated by carefully transecting the telencephalon–diencephalon junction and the total RNA was immediately extracted from the telencephalon with the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA quality (the A260/A280 ratio) and concentration were measured with a NanoDrop spectrophotometer and the samples were stored at −75 °C until further processing. From each sample, an estimated 100 ng of total RNA was reverse-transcribed to cDNA in an Eppendorf Mastercycler pro S using the iScript cDNA Kit (Bio-Rad) according to the manufacturer's instructions. Three embryos were used from each dam.

4.4. *Quantitative RT-PCR analysis*

The quantitative (real-time) reverse-transcription polymerase chain reaction (qRT-PCR) analysis was based on our published protocol (Hernandez and Janušonis, 2010). Five mRNAs of interest (the four 5-HT_4R splice variants and β_2AR) and three reference (housekeeping) mRNAs (β_2-microglobulin, glyceraldehyde-3-phosphate dehydrogenase, and TATA box binding protein) were analyzed. The amplification was performed in a MyiQ single color real-time PCR detection system (Bio-Rad). Each PCR reaction (20 μL) was performed in triplicate and contained the cDNA equivalent of 20 ng RNA, forward and reverse splice variant-specific primers (0.5 μM each; Integrated DNA Technologies, Inc.), 0.2 mM dNTPs, 0.25 U Platinum Taq DNA polymerase (Invitrogen), 0.7X SYBR Green I, and 10 nM fluorescein in a PCR buffer containing 10 mM Tris–HCl (pH 9.0 at 25 °C), 50 mM KCl, 2.5 mM $MgCl_2$, and 0.1% Triton X-100. The primers were designed in Beacon Designer (Premier Biosoft International) and are given in Table 2. The amplification conditions were as follows: 94 °C (10 min); 45 cycles of 94 °C (10 s), 56 °C (10 s), 72 °C (90 s), 78 °C (20 s), 82 °C (20 s); 95 °C (1 min); and 60 °C (1 min). In order to minimize non-specific signal, fluorescence values used in the analysis were obtained at the highest available temperature at

Table 2 – The quantitative RT-PCR primers and the amplification efficiencies (since amplification efficiencies depend only on primers and amplification conditions, all experimental conditions were used to obtain the estimates). Abbreviations: bp, amplicon length in base pairs; T, product analysis temperature; E, mean amplification efficiency; SEM, standard error of the mean.

Target mRNA accession	Target mRNA	Forward primer (5′-3′)	Reverse primer (5′-3′)	bp	T (°C)	E ± SEM
Y09587	5-HT_4R(a)	ATCCTCTGCTGTGATGATGAG	ACTGTGCAAAACTGTATACCTTAG	120	82	1.696 ± 0.006
Y09585	5-HT_4R(b)	CCTGGACAATGACCTAGAAGAC	TTGCCTCTGCTCTTGGAAAG	121	82	1.734 ± 0.006
Y09588	5-HT_4R(e)	ATCCTCTGCTGTGATGATGAG	GGAACAGGTCTATTGCGGAAG	134	82	1.713 ± 0.005
AJ011369	5-HT_4R(f)	ACCTGTTCCCGTCTAACTGAG	TAGTAACCTGTTCATGCAGACAC	190	82	1.766 ± 0.005
NM_007420	β_2AR	TCTGTCTGTCTGTCTGGATGATG	CCCATTGTCACAGCAGAAAGG	167	78	1.731 ± 0.005
NM_009735 (reference gene)	β_2-Microglobulin (B2M)	GGAGAATGGGAAGCCGAACATAC	AGAAAGACCAGTCCTTGCTGAAG	143	78	1.812 ± 0.005
NM_008084 (reference gene)	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AATGTGTCCGTCGTGGATCTGA	AGTGTAGCCCAAGATGCCCTTC	117	82	1.794 ± 0.005
NM_013684 (reference gene)	TATA box binding protein (TBP)	GTTGGTGATTGTTGGTTTTAAGGG	GGAAGGCGGAATGTATCTGG	197	78	1.801 ± 0.005

which no melting of the product double-stranded DNA was detected. Controls with no reverse transcription indicated no contamination with genomic DNA.

The efficiency of each amplification was calculated automatically by using a Mathematica (Wolfram Research, Inc.) program (Hernandez and Janušonis, 2010) based on a published algorithm (Tichopad et al., 2003) with modifications. Briefly, linear regression was used to model the initial baseline of the non-transformed fluorescence data, and the beginning of the exponential phase was defined as the amplification cycle in which the first of three consecutive regression outliers was detected (the outliers were defined as points whose externally studentized residual was significant at the 0.005 level). The exponential phase was modeled by linear regression of the log-transformed fluorescence data and the end of the exponential phase was defined as the amplification cycle in which the first regression outlier was detected (the outlier was defined as the first point whose externally studentized residual was significant at the 0.10 level). The mRNA amount in the initial tissue sample was calculated as the triplicate mean of $(RFU - baseline)E^{-x}$, where *baseline* is the mean baseline fluorescence before the exponential phase, x and RFU are the cycle number and the fluorescence at the mid-point of the exponential phase, and E is the amplification efficiency of the mRNA species. The normalized mRNA amount was calculated by dividing the mRNA amount by the geometric mean of the mRNA amounts of the reference genes in the same sample (Vandesompele et al., 2002). For convenience, the obtained value was multiplied by 1000.

4.5. *Statistical analysis*

Statistical analyses were performed in R 2.14 (The R Foundation for Statistical Computing) and in IBM SPSS 19 (IBM, Inc.). Mixed-effects models were analyzed with the R *nlme* package (Pinheiro et al., 2011) and non-homogeneity of variances was accounted for by choosing the *varIdent* variance structure (Zuur et al., 2009). These approaches are superior to traditional statistical tests that are based on mathematical convenience rather than biological relevance and are strongly recommended for experimental research in neuroscience (Lazic, 2010; Nakagawa and Hauber, 2011). By following a well-established procedure (Zuur et al., 2009), statistical models were built sequentially by using restricted maximum likelihood estimation (REML) and maximum likelihood estimation (ML): (*i*) first, a "beyond optimal" model was constructed that included all relevant fixed effects; (*ii*) the structure of variances and random effects was optimized by comparing nested REML-fitted models with ANOVA and testing the significance of the likelihood ratios (L) (in mixed-effects analyses, the significance of L was calculated "on the boundary"); (*iii*) the structure of fixed effects was optimized by comparing nested ML-fitted models with ANOVA and testing the significance of L (the non-significant fixed effects were removed); (*iv*) the final model was refitted with REML and validated (tested for the normality and homogeneity of residuals). In all tests, the significance level was set at 0.05. For post-hoc analyses, the Bonferroni correction was used.

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