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Quantitative mRNA Analysis of Serotonin 5-HT_4 and Adrenergic β_2 Receptors in the Mouse Embryonic Telencephalon

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Key Words

5-hydroxytryptamine · Autism spectrum disorders · Brain development · Cerebral cortex · Depression · Nested genes · Norepinephrine · Quantitative real-time RT-PCR · Stress

Abstract

The adrenergic β_2 receptor (β_2-AR) gene is embedded (nested) within the serotonin 5-HT_4 receptor (5-HT_4-R) gene and these two receptors can interact at the transcriptional and post-transcriptional levels. The mouse 5-HT_4-R gene contains a number of exons and codes at least four mRNA splice variants (5-$HT_{4(a)}$-R, 5-$HT_{4(b)}$-R, 5-$HT_{4(e)}$-R, 5-$HT_{4(f)}$-R), whereas the β_2-AR gene is intronless. Since 5-HT_4-Rs and β_2-ARs can form homodimers and heterodimers and they increase intracellular cAMP levels, these receptors may be important for integrating serotonergic and noradrenergic signals at the single-neuron level. Both 5-HT_4-R and β_2-AR have been implicated in autism spectrum disorders, depression, and Alzheimer's disease. In the fetal brain, these receptors may mediate the effects of stress on neurodevelopmental processes. We used quantitative reverse-transcription PCR (qRT-PCR) to investigate the developmental expression of 5-HT_4-R and β_2-AR in the mouse telencephalon at embryonic days (E) 13–18. At E13–E14, the mRNA levels of all 5-HT_4-R splice variants were very low, but by E17–E18 they increased 7-fold (5-$HT_{4(a)}$-R), 5-fold (5-$HT_{4(b)}$-R), 9-fold (5-$HT_{4(e)}$-R), and 11-fold (5-$HT_{4(f)}$-R). The expression of 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R was rapidly upregulated between E14 and E15, at the time when the thalamocortical projections arrive in the telencephalon. This pattern was not observed in the expression of 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R, the mRNA levels of which showed a steady, gradual increase from E13 to E18. The β_2-AR mRNA levels were relatively high throughout the studied period of development and increased only by 70% from E13–E14 to E17–E18. These findings suggest that 5-HT_4-R splice variants and β_2-ARs are differentially regulated in the embryonic telencephalon and that their relative amounts may carry developmentally important information.

Introduction

The serotonin 5-HT_4 receptor (5-HT_4-R) is coded by a complex gene that is expressed in the central and enteric nervous systems [Bockaert et al., 2004; Liu et al., 2005; Vilaro et al., 2005; Gershon and Tack, 2007] and in other organs [Claeysen et al., 1999; Medhurst et al., 2001]. The 5-HT_4-R gene contains a large number of exons (at least 38 in humans) and produces multiple mRNA splice vari-

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ants with a long 5′-untranslated region (5′-UTR) [Bockaert et al., 2004; Maillet et al., 2005; Bockaert et al., 2006]. Several lines of evidence suggest that 5-HT_4-Rs interact with adrenergic β_2 receptors (β_2-ARs) and that these interactions may be functionally important for the developing and adult brain.

The β_2-AR gene is embedded (nested) within a 5′-UTR intron region of the host 5-HT_4-R gene [Bockaert et al., 2004] and the expression of these two receptors may be interdependent at the transcriptional level [Henikoff and Eghtedarzadeh, 1987; Gibson et al., 2005; Kumar, 2009] (fig. 1a). Both 5-HT_4-Rs and β_2-ARs increase cAMP levels in neurons [Bockaert et al., 2004] and they can form homodimers and heterodimers [Berthouze et al., 2005; Kamel et al., 2005; Berthouze et al., 2007]. Therefore, 5-HT_4-Rs and β_2-ARs are well-positioned to integrate serotonergic and noradrenergic signals at the single-cell or subcellular level. The possibility of such integration is further supported by the evidence that β_2-ARs and AMPA-type glutamate receptor GluR1 subunits can form functional complexes [Joiner et al., 2010].

The 5-HT_4-R gene produces at least ten *C*-terminal mRNA splice variants in humans and pigs [Bockaert et al., 2006; De Maeyer et al., 2008; Ray et al., 2009] and four splice variants in mice [Bockaert et al., 2004]. The mouse splice variants (5-$HT_{4(a)}$-R, 5-$HT_{4(b)}$-R, 5-$HT_{4(e)}$-R, 5-$HT_{4(f)}$-R) exhibit high constitutive (5-HT-independent) activity and the shorter 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R variants are more constitutively active than the longer 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R variants [Claeysen et al., 1999; Pellissier et al., 2009]. In contrast, the β_2-AR mRNA undergoes no alternative splicing and β_2-ARs exhibit low constitutive activity [Claeysen et al., 1999]. Upon agonist stimulation, both 5-HT_4-Rs and β_2-ARs can be desensitized via the β-arrestin pathway [Lohse et al., 1990; Barthet et al., 2005]; however, 5-HT_4-Rs are typically internalized with no recycling, whereas β_2-ARs are rapidly recycled to the cell membrane following internalization [Barthet et al., 2005]. A recent study has shown that 5-HT_4-R internalization and recycling may be splice-variant specific [Mnie-Filali et al., 2010]. These data suggest that, at least in theory, the strength and temporal dynamics of cAMP signaling in a neuron can be finely regulated by the availability of 5-HT_4-R splice variants, β_2-ARs, and their complexes.

The developing rodent forebrain exhibits a highly dynamic pattern of 5-HT_4-R expression [Waeber et al., 1994, 1996; Slaten et al., 2010], suggesting that factors interfering with this pattern may lead to functional brain abnormalities. Since 5-HT_4-Rs receptors may interact with β_2-ARs during embryonic and fetal development and β_2-ARs can detect stress-related signals, the ontogeny of 5-HT_4-R and β_2-AR levels in the developing forebrain has potentially important implications for understanding autism spectrum and other developmental disorders [Cheslack-Postava et al., 2007; Vincent et al., 2009; Yirmiya and Charman, 2010]. In the present study, we used quantitative reverse-transcription polymerase chain reaction (qRT-PCR) to measure the expression levels of all 5-HT_4-R splice variants and β_2-AR in the embryonic mouse telencephalon from embryonic day 13 to birth.

Materials and Methods

Collection of Tissue

Timed-pregnant CD-1 mice were purchased from Charles River Laboratories, and were housed in individual cages on a 12:12 light-dark cycle (lights on at 07:00, off at 19:00). All experiments were approved by the UCSB Institutional Animal Care and Use Committee.

The levels of nine mRNAs (table 1) were analyzed at 6 embryonic days (E) (E13–E18). Pregnant mice were terminally anesthetized with an overdose of 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 on ice. Embryos were removed from the uterus, decapitated, and their brains were dissected with a fine forceps under a stereoscope. The telencephalon (fig. 1b) was isolated by carefully transecting the telencephalon-diencephalon junction with a Dumont No. 5 forceps. 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. Four embryos were analyzed at each developmental time-point (none of the collected samples overlapped with those used in our previous study [Slaten et al., 2010]). This experimental design allowed us to achieve good temporal resolution (every embryonic day was analyzed in the E13–E18 interval), at the cost of larger errors of the mean at each of the time-points. In order to reduce these errors, we used an advanced computational algorithm [Tichopad et al., 2003] to extract accurate information from each qPCR amplification and screened the data for outliers.

Reverse Transcription and qPCR Amplification

From each sample, 100 ng of total RNA was reverse-transcribed to cDNA in a PxE thermal cycler (ThermoFisher Scientific) using the iScript cDNA Kit (Bio-Rad) according to the manufacturer's instructions. Quantitative PCR analysis of the four 5-HT_4-R mRNA splice variants (5-$HT_{4(a)}$-R, 5-$HT_{4(b)}$-R, 5-$HT_{4(e)}$-R, 5-$HT_{4(f)}$-R), the β_2-AR mRNA, and the four housekeeping (reference) mRNAs 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), 0.2 mM dNTPs, 0.25 U

Table 1. Quantitative RT-PCR primers used in the study

Target mRNA accession	Target mRNA	Forward primer (5′–3′)	Reverse primer (5′–3′)	bp	T °C	E
Y09587	5-$HT_{4(a)}$	ATCCTCTGCTGTGATGATGAG	ACTGTGCAAAACTGTATACCTTAG	120	82	1.623
Y09585	5-$HT_{4(b)}$	CCTGGACAATGACCTAGAAGAC	TTGCCTCTGCTCTTGGAAA	121	82	1.674
Y09588	5-$HT_{4(e)}$	ATCCTCTGCTGTGATGATGAG	GGAACAGGTCTATTGCGGAAG	134	82	1.672
AJ011369	5-$HT_{4(f)}$	ACCTGTTCCCGTCTAACTGAG	TAGTAACCTGTTCATGCAGACAC	190	82	1.671
NM_007420	β_2-AR	TCTGTCTGTCTGTCTGGATGATG	CCCATTGTCACAGCAGAAAGG	167	78	1.616
NM_009735 (housekeeping gene)	β_2-microglobulin (B2M)	GGAGAATGGGAAGCCGAACATAC	AGAAAGACCAGTCCTTGCTGAAG	143	78	1.719
NM_008084 (housekeeping gene)	glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	AATGTGTCCGTCGTGGATCTGA	AGTGTAGCCCAAGATGCCCTTC	117	82	1.788
NM_019639 (housekeeping gene)	ubiquitin C (UBC)	GATCTTTGCAGGCAAGCAGCT	TTCTCTATGGTGTCACTGGGCTC	174	82	1.751
NM_013684 (housekeeping gene)	TATA box binding protein (TBP)	GTTGGTGATTGTTGGTTTAAGGG	GGAAGGCGGAATGTATCTGG	197	78	1.696

bp = Product length in base pairs; T = product analysis temperature; E = mean amplification efficiency.

Platinum Taq DNA polymerase (Invitrogen), 0.7× 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 1. 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); 60°C (1 min); and the product melting curves were obtained by ramping temperature from 60 to 95°C in 0.5°C increments (fig. 1c). In order to minimize the non-specific signal, fluorescence values used in the analysis were obtained at the highest available temperature at which no melting of the product double-stranded DNA was detected. Due to very low levels of some 5-HT_4-R mRNA splice variants (especially at E13–E14), non-specific products (such as primer-dimers) were detected in some amplifications. The identity of these non-specific products was confirmed in control amplifications with no template. Amplifications that yielded only non-specific products or non-specific products with melting temperatures higher than the analysis temperature were eliminated from the triplicate. 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 (online suppl. file, www.karger.com/doi/10.1159/000314723) 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 0.01 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). This fast algorithm reliably isolated the exponential phase in most PCR amplifications, including samples with low mRNA levels (fig. 1d). 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 in the middle point of the exponential phase, and *E* is the mean efficiency of all amplifications (at all embryonic ages) of the particular mRNA. For convenience, the obtained value was multiplied by 10^6. The normalized mRNA amount was calculated by dividing the mRNA amount by the geometric mean of the mRNA amounts of verified housekeeping genes in the same sample [Vandesompele et al., 2002].

Statistical analyses were performed in SPSS 17.0 (SPSS, Inc.) with the significance level set at 0.05. In post-hoc comparisons, the Tukey HSD test was used.

Results

Validation of Housekeeping Genes

The housekeeping genes selected for this study are commonly used as internal references for qRT-PCR normalization. However, the expression stability of these genes is not guaranteed and several approaches can be used to verify them in each experimental design [Vandesompele et al., 2002; Pfaffl et al., 2004; Bustin and Nolan, 2009]. Since we attempted to load the same amount of total mRNA in each reverse-transcription reaction, the non-normalized mRNA amounts of the four housekeeping genes were used to assess the expression stability of the genes during the studied developmental period (fig. 2). All four genes showed no significant changes as

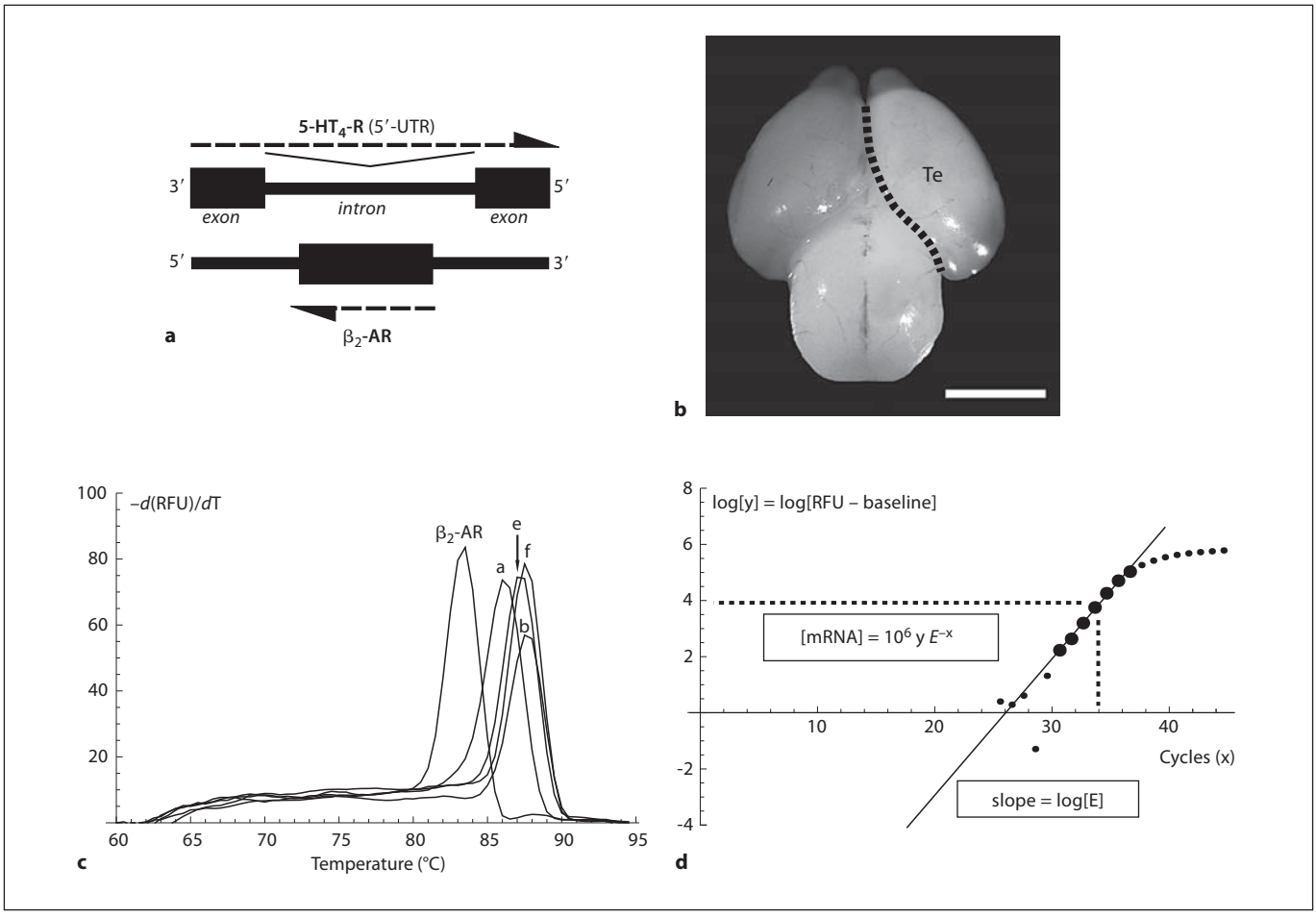

Fig. 1. Quantitative mRNA analysis of 5-HT_4-R and β_2-AR. **a** The β_2-AR gene is nested in an intron region of the 5-HT_4-R 5′-untranslated region (5′-UTR). The exons and intron are not drawn to scale. **b** The embryonic brain region (the telencephalon, Te) used in the analysis. An E17 brain is shown. Scale bar = 2 mm. **c** The melting curves of the PCR amplification products (the mRNA is indicated above the peaks). All of the shown curves were obtained from the same E18 sample. **d** A representative baseline-subtracted log-transformed amplification curve and the automatic detection of the exponential phase (filled circles; see 'Materials and Methods' and the online suppl. file). The figure shows an actual 5-$HT_{4(a)}$-R amplification of a sample obtained from an E13 telencephalon.

determined by ANOVA (table 2), but the expression of β_2-microglobulin yielded a low p value due to an apparent two-fold increase in expression at E18 (fig. 2b). We next pooled all embryonic ages and calculated the cross-correlations between the non-normalized mRNA amounts of the genes (table 2). With the exception of β_2-microglobulin, all cross-correlations between the pairs of the remaining housekeeping genes were highly significant, further validating them as reference genes. We also found that β_2-microglobulin yielded the largest gene instability measure *M* [Vandesompele et al., 2002] (table 2).

Based on these findings, β_2-microglobulin was removed from the housekeeping gene set and mRNA amounts were normalized by the geometric mean of the mRNA amounts of the remaining three housekeeping genes [Vandesompele et al., 2002].

Data Screening

Since the copy numbers of 5-HT_4-R mRNAs are relatively low in the embryonic brain, calculated mRNA amounts are expected to be sensitive to stochastic noise in the RNA isolation and reverse transcription steps [Tichopad et al., 2009; Kitchen et al., 2010], as well as to the Monte Carlo effect in the PCR amplification step [Bustin and Nolan, 2009]. Therefore, the obtained mRNA amounts at each developmental point were screened for

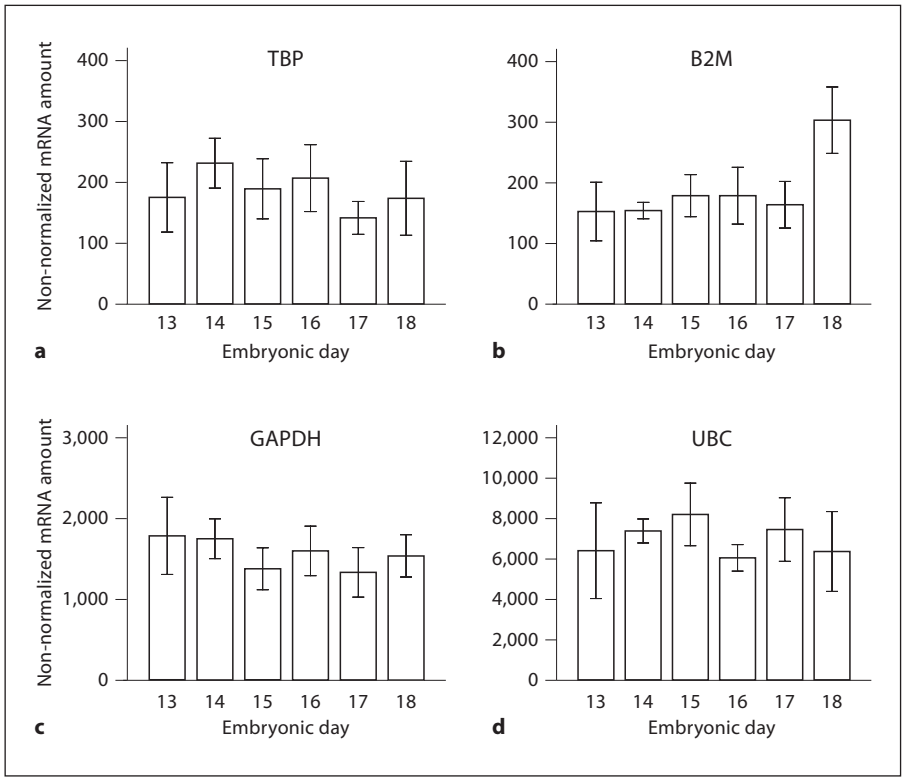

Fig. 2. The non-normalized mRNA amounts of the four preselected housekeeping genes. The error bars are the standard errors of the mean.

Table 2. Analysis of the housekeeping genes

	ANOVA			Correlations			*M*
	F	d.f.	p	B2M	GAPDH	UBC	
TBP	0.386	5, 18	0.852	0.372 0.073 24	0.739** $4 \cdot 10^{-5}$ 24	0.785** $6 \cdot 10^{-6}$ 24	0.706
B2M	1.898	5, 18	0.145	1	0.533** 0.007 24	0.295 0.162 24	0.858
GAPDH	0.340	5, 18	0.882		1	0.642** 0.001 24	0.675
UBC	0.272	5, 18	0.922			1	0.758

ANOVA was used to test whether the non-normalized mRNA amounts of the selected housekeeping genes were stable at different embryonic ages. Next, all embryonic ages were pooled and Pearson's correlation coefficients were calculated between the non-normalized mRNA amounts of the four genes. In the correlation cells, the first number is the correlation coefficient; the second number is the two-tailed p value; and the third number is the sample size. Also, the gene expression instability measure *M* [Vandesompele et al., 2002] was calculated. The obtained results suggest that β_2-microglobulin (B2M) should be removed from the reference gene set. d.f. = Degrees of freedom; ** $p < 0.01$.

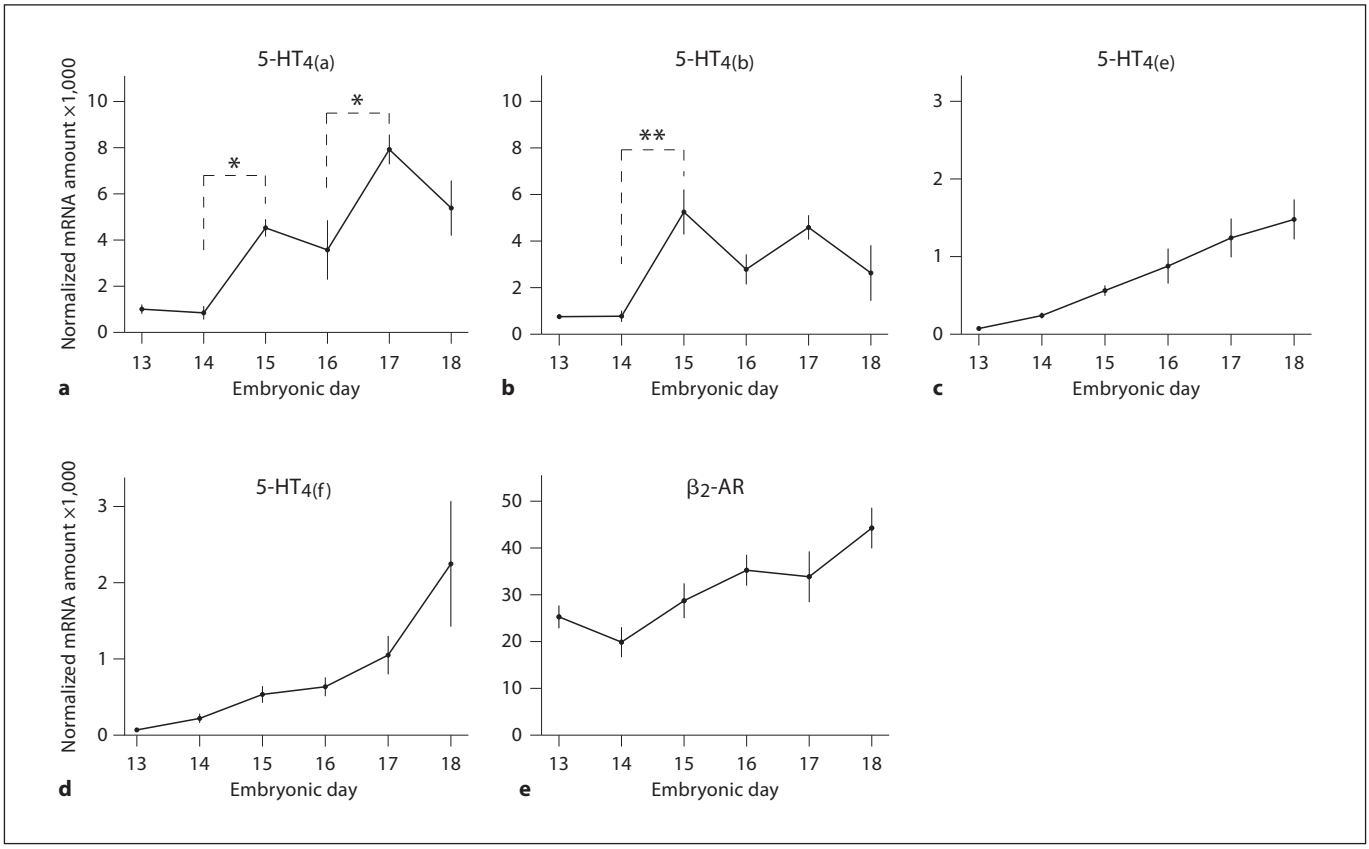

Fig. 3. The normalized mRNA amounts of the four 5-HT_4-R splice variants and β_2-AR. The error bars are SEM. The dashed lines indicate all significant post-hoc (Tukey HSD) differences between consecutive time points. * $p < 0.05$, ** $p < 0.01$.

outliers by calculating the two-tailed p value of Dixon's Q parameter [Dixon, 1951; Rorabacher, 1991; Efstathiou, 2006]. At E16, an outlier (the normalized mRNA amount = 2.8; $p < 0.05$) was found in the $HT_{4(f)}$-R set; this case was excluded from further analyses of the 5-$HT_{4(f)}$-R splice variant. At E17, an extreme outlier was found in the 5-$HT_{4(a)}$-R set (the normalized mRNA amount = 38.9; $p < 0.01$). Since this same case had the highest mRNA levels of all 5-HT_4-R splice variants, it was excluded from further analyses of all 5-HT_4 splice variants. At E18, one case was found to be an outlier in the 5-$HT_{4(a)}$ set (the normalized mRNA amount = 41.3; $p < 0.05$) and it was a near-outlier in the 5-$HT_{4(e)}$ set (the normalized mRNA amount = 4.7; $p = 0.08$); this case was excluded from further analyses of all 5-HT_4 splice variants. Consistent with a recent methodological study of qRT-PCR variability [Kitchen et al., 2010], the outliers showed little variability within the PCR triplicate. No outliers were found in the β_2-AR set.

5-HT_4-R and β_2-AR Levels in the Developing Telencephalon

Overall, the telencephalic mRNA levels of the four 5-HT_4-R splice variants and β_2-AR showed a highly significant increase ($p < 0.01$; table 3) as the brain progressed in its development from E13 to E18 (fig. 3). However, the amounts and patterns of the increase were distinctly different among these mRNAs. At E13–E14, the mRNA levels of all 5-HT_4-R splice variants were very low, but by E17–E18 they increased approximately seven-fold (5-$HT_{4(a)}$-R), five-fold (5-$HT_{4(b)}$-R), nine-fold (5-$HT_{4(e)}$-R), and eleven-fold (5-$HT_{4(f)}$-R). The post-hoc analysis of the four 5-HT_4-R splice variants revealed three different patterns of mRNA increase. The mRNA levels of 5-$HT_{4(a)}$-R were rapidly upregulated at two developmental times: between E14 and E15 ($p = 0.033$) and between E16 and E17 ($p = 0.018$). The mRNA levels of 5-$HT_{4(b)}$-R were rapidly upregulated only once, between E14 and E15 ($p = 0.002$). The mRNA levels of 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R

Table 3. ANOVA of the embryonic age effect on the telencephalic mRNA levels of the four 5-HT_4-R splice variants and β_2-AR

	F	d.f.	p	η^2
5-$HT_{4(a)}$	10.60	5, 15	0.0002	0.779
5-$HT_{4(b)}$	7.87	5, 16	0.0007	0.711
5-$HT_{4(e)}$	8.69	5, 14	0.0006	0.756
5-$HT_{4(f)}$	5.77	5, 14	0.0043	0.673
β_2-AR	5.00	5, 17	0.0053	0.595

d.f. = Degrees of freedom; η^2 = effect size.

Table 4. Partial correlations (controlling for embryonic age) between the mRNA amounts of the four 5-HT_4-R splice variants and β_2-AR

	5-$HT_{4(b)}$	5-$HT_{4(e)}$	5-$HT_{4(f)}$	β_2-AR
5-$HT_{4(a)}$	0.873** $1 \cdot 10^{-5}$	0.576 0.020	0.108 0.691	–0.032 0.905
5-$HT_{4(b)}$		0.402 0.123	–0.051 0.852	0.193 0.474
5-$HT_{4(e)}$			0.721* 0.002	0.203 0.450
5-$HT_{4(f)}$				0.387 0.138

By taking into account the Bonferroni correction for the ten cross-correlations, the significance level was set at 0.005.

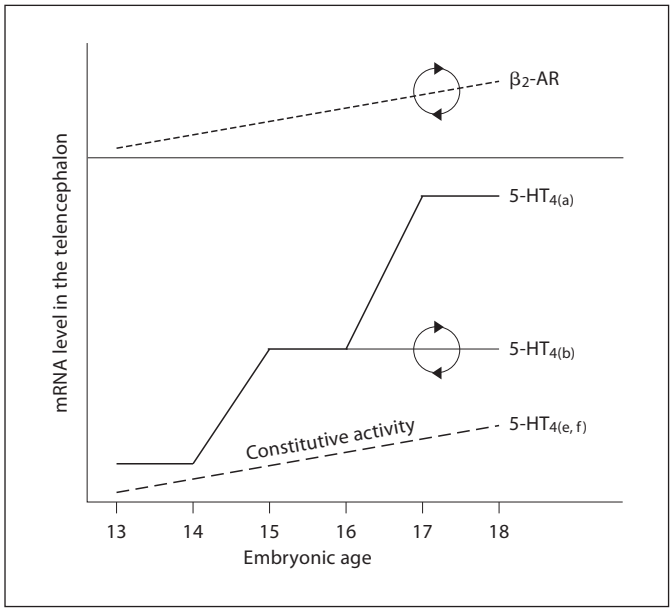

Fig. 4. The hypothesized dynamics of mRNA expression in the developing telencephalon at E13–E18. The circles mark the receptors that have been shown to undergo recycling to the membrane after internalization [Barthet et al., 2005; Mnie-Filali et al., 2010]. Theoretically, these receptors can be functionally 'up-regulated' or 'down-regulated' with no changes in transcription and translation and, therefore, these shifts may not be reflected in their mRNA levels.

showed an almost linear increase during the studied period of development and the post-hoc analysis revealed no significant differences between any two consecutive time points.

In contrast to the 5-HT_4-R splice variants, relatively high β_2-AR mRNA levels were detected as early as E13 and the mRNA levels at E17–E18 were only approximately 70% higher than those at E13–E14 (fig. 3e). The β_2-AR mRNA levels showed an almost linear increase during the studied period of development and the post-hoc analysis revealed no significant differences between any two consecutive time points.

No significant partial correlations (controlling for embryonic age) were found between the β_2-AR mRNA levels and the mRNA levels of each of the 5-HT_4-R splice variants ($p > 0.1$; table 4). Significant partial correlations (after the Bonferroni correction) were found between the 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R splice variants ($p = 10^{-5}$) and between the 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R splice variants ($p = 0.002$).

Discussion

The obtained results are summarized in figure 4. Compared to the 5-HT_4-R mRNA levels, the β_2-AR mRNA levels remained relatively high and stable throughout the studied developmental period. The relatively high β_2-AR mRNA expression is consistent with a general observation in molecular biology that the nested gene is typically expressed at higher levels than the host gene [Castillo-Davis et al., 2002; Gibson et al., 2005]. While the β_2-AR mRNA levels increased significantly from E13–E14 to E17–E18, the increase was only approximately 70%. In contrast, at E13–E14 the mRNA levels of all four 5-HT_4-R splice variants were very low, but by E17–E18 they increased five- to eleven-fold.

The functional significance of the high β_2-AR expression in the telencephalon as early as E13 is intriguing because, in the rodent brain, the noradrenergic afferents from the locus coeruleus reach the developing cerebral cortex only around E17 [Levitt and Moore, 1979; Verney et al., 1984] and β_2-ARs exhibit relatively low levels of constitutive activity [Claeysen et al., 1999]. Therefore, the β_2-AR mRNA may remain untranslated, or β_2-ARs may be synthesized but remain inactive. Also, the early β_2-ARs may detect epinephrine and norepinephrine signals originating outside the central nervous system [Newnham et al., 1984; Lenard et al., 2003; Ebert et al., 2008] before the blood-brain barrier matures, and may mediate their effects on the developing brain. Interestingly, an early study has found detectable norepinephrine levels in the mouse cerebral cortex at E14 [Elias et al., 1982]. It has been suggested that prenatal stressors may play a role in autism spectrum disorders, schizophrenia, depression, and other developmental disturbances [Diego et al., 2006; O'Donnell et al., 2009; Yirmiya and Charman, 2010].

The developmental expression pattern of 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R suggests that these splice variants are rapidly upregulated between E14 and E15, which coincides with the time when the thalamocortical projections enter the telencephalon [Molnar et al., 1998]. We have previously shown that the thalamocortical projections express 5-HT_4-Rs in the embryonic mouse brain [Slaten et al., 2010]. However, it is unlikely that the observed upregulation was due to an increase in the mRNA levels in thalamocortical neurons, since our samples did not include the diencephalon and long-projection axons typically do not contain mRNA [Vilaro et al., 2005]. The increase was probably caused by elevated transcription of 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R in young telencephalic neurons, which does not rule out the possibility that this event may be controlled by the arrival of the thalamocortical afferents.

The expression of 5-$HT_{4(a)}$-R (but not 5-$HT_{4(b)}$-R) was again rapidly upregulated between E16 and E17, which coincides with the time when the serotonergic projections from the midbrain raphe complex reach the developing cerebral cortex [Lidov and Molliver, 1982; Wallace and Lauder, 1983; Bruning et al., 1997; Janušonis et al., 2004] and when gliogenesis begins [Liu et al., 2002; Miyata et al., 2010]. It suggests that 5-$HT_{4(a)}$-Rs are highly responsive to developmental serotonin signals and/or that they may be expressed in young glial cells. There is evidence that 5-HT_4-Rs can be expressed in astrocytes [Parga et al., 2007]. It should be noted that our results do not rule out the possibility that signaling through 5-$HT_{4(b)}$-Rs can also be increased at E16–E17. This increase may not be detectable at the mRNA level, since it has been recently shown that this splice variant can undergo recycling to the membrane after internalization [Mnie-Filali et al., 2010] (fig. 4).

The expression of 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R showed a steady, nearly-linear increase over the studied developmental period. Since these splice variants exhibit high constitutive activity [Claeysen et al., 1999], this increase may be independent of the presence of serotonin in the telencephalon and may be important for setting cAMP production levels at the appropriate baseline. It would be interesting to see if this pattern of expression persists if the embryonic serotonin system is pharmacologically or genetically ablated.

Significant correlations (controlling for embryonic age) were found between the mRNA levels of 5-$HT_{4(a)}$-R and 5-$HT_{4(b)}$-R and between 5-$HT_{4(e)}$-R and 5-$HT_{4(f)}$-R, suggesting that the production of each pair of the splice variants may be controlled by the same developmental factors. While no significant correlation was found between the mRNA levels of β_2-AR and each of the 5-HT_4-R splice variants, this finding should be considered inconclusive due to the relatively low statistical power of this analysis (four cases were used at each developmental time-point).

The nested arrangement of the β_2-AR gene in the 5-HT_4-R gene reflects the emerging complexity of the mammalian genome. In humans, at least 158 protein-coding genes (0.5% of the total genome) are nested in introns of other genes [Yu et al., 2005; Kumar, 2009]. To date, 55 nested gene emergence events have been documented in the human genome and no losses of nested gene arrangements have been reported in the studied vertebrates [Assis et al., 2008; Kumar, 2009]. While it is unclear how many neurotransmitter genes are nested in other neurotransmitter genes, the human serotonin 5-HT_{2B} receptor gene is nested in the gene coding a proteasome 26S subunit (PSMD1) [Yu et al., 2005]. The number of nested genes may further increase as genes with long untranslated regions become correctly annotated [Yu et al., 2005]. Nested genes have been shown to code proteins important for invertebrate development [Henikoff and Eghtedarzadeh, 1987], but virtually nothing is known about their role in the developing mammalian brain. It also unclear to what extent the expression of the host gene typically affects the expression of the nested gene, and vice versa. While one study has found statistically equal correlations between the expression levels of nested and simply adjacent gene pairs [Assis et al., 2008], other authors have emphasized that the transcription of the nested and host genes can be regulated by common enhancers, steric interactions between the two RNA polymerase complexes (which have to pass each oth-

er), and other events [Henikoff and Eghtedarzadeh, 1987; Gibson et al., 2005]. Collisions between two RNA polymerase complexes moving in the opposite directions and their subsequent stalling have now been visualized by atomic force microscopy [Crampton et al., 2006].

In the adult brain, 5-HT_4-Rs and β_2-ARs are abundant in the amygdala [Vilaro et al., 2005; Qu et al., 2008; Abraham et al., 2008] and in the pyramidal neurons of the hippocampus [Davare et al., 2001; Vilaro et al., 2005], which suggests that their interaction may be relevant to a number of neurological disorders. Studies have shown that 5-HT_4-Rs and β_2-ARs may play important roles in the neurobiology of depressive disorders [Mongeau et al., 1997; Sastre et al., 2001; Duman, 2007; Lucas et al., 2007; Tamburella et al., 2009], Alzheimer's disease [Lezoualc'h, 2007; Yu et al., 2008, 2010], and autism spectrum disorders [Connors et al., 2005; Cheslack-Postava et al., 2007; Janušonis, 2008; Vincent et al., 2009; Slaten et al., 2010].

In summary, our results provide new information about the role of nested neurotransmitter genes in brain development. They indicate that the 5-HT_4-R splice variants and β_2-AR are differentially regulated in the embryonic telencephalon and that this interaction may play a significant role in developmental brain disorders, including those in which prenatal stress is a risk factor.

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