

Ontogeny of brain and blood serotonin levels in 5-HT_{1A} receptor knockout mice: potential relevance to the neurobiology of autism

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Abstract

The most consistent neurochemical finding in autism has been elevated group mean levels of blood platelet 5-hydroxytryptamine (5-HT, serotonin). The origin and significance of this platelet hyperserotonemia remain poorly understood. The 5-HT_{1A} receptor plays important roles in the developing brain and is also expressed in the gut, the main source of platelet 5-HT. Post-natal tissue levels of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA) and tryptophan were examined in the brain, duodenum and blood of 5-HT_{1A} receptor-knockout and wild-type mice. At 3 days after birth, the knockout mice had lower mean brain 5-HT levels and normal mean platelet 5-HT levels. Also, at 3 days after birth, the mean tryptophan levels in the

brain, duodenum and blood of the knockout mice were around 30% lower than those of the wild-type mice. By 2 weeks after birth, the mean brain 5-HT levels of the knockout mice normalized, but their mean platelet 5-HT levels became 24% higher than normal. The possible causes of these dynamic shifts were explored by examining correlations between central and peripheral levels of 5-HT, 5-HIAA and tryptophan. The results are discussed in relation to the possible role of 5-HT in the ontogeny of autism.

Keywords: autism, blood platelets, brain development, hyperserotonemia, 5-hydroxytryptamine (serotonin), 5-HT_{1A}. *J. Neurochem.* (2006) **99**, 1019–1031.

Autism spectrum disorder is a pervasive developmental disorder with the current estimated incidence rate of around 30–60 cases per 10 000 (Rutter 2005). Despite decades of research, the biological causes of autism spectrum disorder remain poorly understood. A wide range of brain abnormalities has been reported in autistic individuals (Waterhouse *et al.* 1996; Courchesne 1997; Casanova *et al.* 2002; Sparks *et al.* 2002; Palmen *et al.* 2004), but at least some of these purported abnormalities may be the consequence of earlier alterations of brain development (Bauman and Kemper 2005). Such pre-natal or early post-natal alterations may be difficult to detect in the brains of older individuals examined after autism has been diagnosed (around 2 or 3 years of age).

Since the early 1960s, the most consistent biological finding in autistic individuals has been their elevated group mean levels of 5-hydroxytryptamine (5-HT, serotonin) in the blood platelets (Schain and Freedman 1961; Anderson *et al.* 1990; Anderson 2002). This platelet hyperserotonemia has been reported in various ethnic groups (Schain and Freedman 1961; McBride *et al.* 1998; Mulder *et al.* 2004), further suggesting that this phenomenon is general and may be important in understanding the neurobiological etiology of autism. Unfortunately, it remains unclear what causes the hyperserotonemia of autism and whether it develops pre-

nately or post-natally. Clarification of these important issues may be critical to illuminating the relationship between the platelet hyperserotonemia and the developing brain. It may also guide the design of animal models (Kahne *et al.* 2002).

The 5-HT found in blood platelets is derived from the 5-HT synthesized by gut enterochromaffin (EC) cells, the main source of peripheral 5-HT (Anderson *et al.* 1987; Walther *et al.* 2003; Gershon 2004). The 5-HT released by EC cells serves as a neurotransmitter in the enteric nervous system (Gershon 2003), but some of the gut 5-HT also enters the blood circulation. Most of this 5-HT is rapidly cleared by the liver, lungs, and other organs (Thomas and Vane 1967; Anderson

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Abbreviations used: DAB, 3,3'-diaminobenzidine; EC, enterochromaffin; 5-HIAA, 5-hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine, serotonin; ISS, internal standard solution; NeuN, neuronal nuclei; NMS, N-methylserotonin; PBS, phosphate-buffered saline; PD, post-natal day; SERT, serotonin transporter; *Tph*, tryptophan hydroxylase; TX, Triton X-100.

et al. 1987), but some of it is taken up by platelets through the action of the membrane serotonin transporter (SERT). Compared with platelet 5-HT levels, the concentration of free 5-HT in the human blood plasma is negligible (Anderson *et al.* 1987; Beck *et al.* 1993). It appears therefore that the hyperserotonemia of autism could be caused by one or more of the following three processes: (i) more 5-HT being released from the gut into the blood circulation; (ii) less 5-HT being cleared from the blood circulation before it is stored in blood platelets; and (iii) more efficient uptake of 5-HT by blood platelets.

In the present study, we examine whether platelet hyperserotonemia may be caused by abnormal regulation of 5-HT release from the gut. We have suggested in a previous study that published clinical data are consistent with this possibility (Janušonis 2005). The control of 5-HT release from EC cells is not completely understood and may depend on several neurotransmitters (Racke and Schworer 1992; Schworer and Ramadori 1998; Christofi *et al.* 2004). Only recently have studies provided more information about what neurotransmitter receptors EC cells actually express (Kirchgessner *et al.* 1996; Schafermeyer *et al.* 2004; Liu *et al.* 2005). Here, we focus on the 5-HT_{1A} receptor, an intronless 5-HT receptor (Lanfumeij and Hamon 2004; Pucadyil *et al.* 2005), that is known to be expressed by EC cells (Kirchgessner *et al.* 1996) and that may regulate 5-HT release from these cells, as suggested by *in vitro* studies (Schworer and Ramadori 1998). In this aspect, the function of gut 5-HT_{1A} receptors may be similar to that of 5-HT_{1A} receptors in the brain, where somatodendritic 5-HT_{1A} autoreceptors may regulate 5-HT release from serotonergic neurons (Adell *et al.* 2002).

In the developing human and rat brain, the expression of 5-HT_{1A} receptors is highly dynamic (Bar-Peled *et al.* 1991; del Olmo *et al.* 1994; Miquel *et al.* 1994). The absence of 5-HT_{1A} receptors in the brain during a critical developmental period results in a mouse phenotype that exhibits increased anxiety-like behavior (Heisler *et al.* 1998; Parks *et al.* 1998; Ramboz *et al.* 1998), even if normal receptor expression is later restored (Gross *et al.* 2002).

We hypothesized that, in autism, the abnormal brain development and the platelet hyperserotonemia may be caused by a common underlying factor that is normally expressed in the developing brain and the gut. In order to test the validity of this general hypothesis, we examined the ontogeny of central and peripheral 5-HT levels in a mouse model that lacks functional 5-HT_{1A} receptors.

Materials and methods

Animals

C57BL/6 mice lacking functional 5-HT_{1A} receptors were generated as described previously (Heisler *et al.* 1998) and were a generous gift of Dr L. H. Tecott (University of California at San Francisco). Heterozygous founders were used to establish a colony and six pairs

of $-/-$ mice were used as breeders to obtain $-/-$ pups. Six $+/+$ females and two $+/+$ males (C57BL/6; Charles River Laboratories Inc., Wilmington, MA, USA) were bred to obtain $+/+$ pups. The genotypes of the pups used in the study were independently confirmed before data analysis. All procedures have been approved by the Yale University Animal Care and Use Committee.

Genotyping

DNA samples were prepared from tails by using Genomic Isolation Kit (Lambda Biotech Inc., St. Louis, MO, USA). The genotypes were determined by PCR using the following primers: 5'-CTGCTCATGCTGGTCCTCTATG-3' (wild-type allele forward primer), 5'-TAGGAGGTAGCTCCTGATTCGC-3' (wild-type allele reverse primer), 5'-CACCTTGCTCCTGCCGAGAAA-3' (Neo D, mutant allele forward primer), and 5'-AGAAGGCGATAGAAGGC-GATG-3' (Neo H, mutant allele reverse primer), which amplified a 250-bp product for the wild-type allele (5-HT_{1A}) and a 500-bp product for the mutant allele (neomycin resistance cassette).

Immunohistochemistry

Adult $+/+$ and $-/-$ mice were perfused with 0.9% saline followed by chilled 4% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M, pH 7.4). The brains were post-fixed in the same fixative overnight at 4°C, cryoprotected in 30% sucrose in PBS for 2 days at 4°C, cut at 40 μ m thickness on a freezing microtome, and stored at -20°C in a cryoprotectant solution containing 30% sucrose, 1% (w/v) polyvinylpyrrolidone, and 30% (v/v) ethylene glycol in PBS.

Neuronal nuclei (NeuN) were labeled by using NeuN immunohistochemistry. Sections were rinsed in PBS, incubated in 0.3% H₂O₂ in PBS for 20 min, rinsed in PBS, blocked in 2% normal donkey serum (NDS; Jackson Immuno-Research Laboratories, West Grove, PA, USA) and 0.3% Triton X-100 (TX) for 1 h, incubated in mouse anti-NeuN IgG₁ (1 : 200; Chemicon, Temecula, CA, USA) with 0.1% TX in PBS overnight at 4°C, rinsed in PBS, incubated in SP-biotin-conjugated donkey anti-mouse IgG (1 : 500, Jackson Immuno-Research Laboratories) with 0.3% TX in PBS for 2 h, rinsed in PBS, incubated in avidin-biotinylated peroxidase complex (1 : 100; Vector Laboratories, Burlingame, CA, USA) in PBS for 1 h, rinsed in PBS, developed in 0.05% 3,3'-diaminobenzidine (DAB) with 0.01% H₂O₂ for 5 min, rinsed in PBS, mounted onto chromium-subbed slides, cleared in xylenes, and coverslipped with Permount.

Serotonergic fibers were labeled by using SERT immunohistochemistry, which was based on a previously published protocol (Donovan *et al.* 2002) with modifications: sections were rinsed in PBS, blocked in 10% NDS and 0.3% TX in PBS for 1 h, incubated in rabbit anti-SERT IgG (1 : 8000; ImmunoStar Corporation, Hudson, WI, USA) in the blocking solution at 4°C for 2 days, rinsed in PBS, incubated in SP-biotin-conjugated donkey anti-rabbit IgG (1 : 200; Jackson Immuno-Research Laboratories) in the blocking solution for 1.5 h, rinsed in PBS, incubated in avidin-biotinylated peroxidase complex (1 : 100) in PBS for 1 h, developed in 0.05% DAB with 0.01% H₂O₂ in PBS for 5 min, rinsed in PBS, mounted onto chromium-subbed slides, and allowed to air-dry. The slides were incubated in 1.42% silver nitrate at 56°C for 1 h, rinsed for 15 min in running deionized water, incubated in 0.2% gold chloride for 10 min in the dark, rinsed in running deionized water for 5 min, incubated in 5% sodium thiosulfate for 5 min, rinsed in running deionized water for 10 min,

dehydrated in a graded series of ethanols, cleared in xylenes, coverslipped with Permount, and viewed in dark field.

Serotonergic cell somata were labeled by using 5-HT immunohistochemistry, which was based on a previously published protocol (Janušonis and Fite 2001) with modifications: sections were rinsed in PBS, incubated in 0.3% H₂O₂ in PBS for 20 min, rinsed in PBS, blocked in 2% normal goat serum (Vector Laboratories) and 0.5% TX in PBS for 1 h, incubated in rabbit anti-5-HT IgG (1 : 1000; Protos Biotech Corporation, New York, NY, USA) with 2% normal goat serum and 2% TX in PBS at 4°C for 2 days, rinsed in PBS, incubated in biotinylated goat anti-rabbit IgG (1 : 200; Vector Laboratories) with 2% normal goat serum and 2% TX in PBS for 1.5 h, rinsed in PBS, incubated in avidin-biotinylated peroxidase complex (1 : 100) for 1 h, rinsed in PBS, incubated in 0.05% DAB with 0.01% H₂O₂ in PBS for 5 min, rinsed in PBS, mounted onto chromium-subbed slides, allowed to air-dry, cleared in xylenes, and coverslipped with Permount.

Tissue preparation for HPLC

Between 08.00 and 12.00 h, 2–4 pups randomly chosen from each litter were killed at post-natal day 3 (PD3) or post-natal day 14 (PD14). Also, young adult (2–4 months) female mice were used. PD3 pups were decapitated and their trunk blood was immediately collected into a drop (25 µL) of 5% Na₂EDTA placed on a piece of parafilm. The blood sample was carefully transferred into a 1.5-mL centrifuge tube and left at room temperature until the dissection of the brain and duodenum was completed. PD14 pups and adult animals were decapitated and their trunk blood was allowed to drip directly into a 1.5-mL centrifuge tube containing 25 µL of 5% Na₂EDTA. The tube was gently vortexed to allow mixing and left at room temperature until the dissection of the brain and duodenum was completed. Following the dissection of the brain and duodenum, 10 µL of the blood sample was diluted with 290 µL of 1 × Dulbecco's PBS (Gibco, Rockville, MD, USA) at room temperature, and this sample was used to obtain automated platelet counts at the Yale–New Haven Hospital Hematology Section. The remaining blood was divided into 20-µL aliquots and stored at –80°C. Samples with blood clots or clearly abnormal platelet counts were excluded from HPLC analysis.

The head was quickly dissected and stored in a pre-weighed 1.5-mL centrifuge tube (PD3) or in a 20-mL scintillation vial (PD14 and adult mice) on dry ice. In the case of PD3 and PD14 pups, a piece of the duodenum, cut from the pylorus of the stomach to around 1.5 cm (PD3) or 2.0 cm (PD14) distal, was stored in a pre-weighed 1.5-mL centrifuge tube on dry ice. The brain and the duodenum were stored at –80°C and the tail was used for genotyping.

HPLC analysis

The brain internal standard solution (ISS) contained 0.1% ascorbic acid, 1% sodium metabisulfite, 1% Na₂EDTA, and 50 ng/mL of the internal standard, *N*-methylserotonin (NMS). Whole PD3 brains and frontal coronal blocks of PD14 and adult brains (from the frontal pole to the level of the mid-striatum) were allowed to thaw on ice and 2 mL, 500 µL, or 1 mL of brain ISS, respectively, was added to the samples. The duodenum ISS contained 3% ascorbic acid, 1% sodium metabisulfite, 114 ng/mL of the internal standard (NMS), and 20% of 3.4 M perchloric acid. PD3 and PD14 duodenum samples were allowed to thaw on ice and 385 or 770 µL of duodenum ISS, respectively, was added to the samples. Each sample

was sonicated for 3–5 s on a Branson sonicator (medium setting). After sonication, 3.4 M perchloric acid (10% v/v) was added to the brain samples. The brain and duodenum samples were kept on ice for 10–15 min and centrifuged in an Eppendorf microcentrifuge at 6000 *g* for 5 min. Approximately 500 µL of the supernate was removed and stored at –80°C until analyzed. Blood samples were prepared by addition of 100 µL of 25% ascorbic acid, 100 µL of 5% sodium metabisulfite, 10 µL of 10 ng/µL NMS (internal standard) to the frozen whole blood sample. After thawing and mixing, 75 µL of 3.4 M perchloric acid was added, samples were kept on ice for 10 min, centrifuged, and the supernate stored at –80°C until analyzed. Brain and duodenum analyses were performed by HPLC with fluorometric detection: a 25 × 0.46 Altex Ultrasphere column heated to 40°C was eluted with a mobile phase consisting of 70% 0.1 M NaH₂PO₄ (pH 4.0) containing 150 mg/L octyl sulfate, 30% methanol. A similar system, differing only in pH (4.7) and methanol content (20%) of the mobile phase, was used for blood analyses. Compounds were detected using a Shimadzu RF10-AXI fluorometer, with excitation and emission wavelengths of 285 and 345 nm, respectively, quantitated by comparing peak heights ratios (analyte/NMS) to those observed for standards, and concentrations expressed as ng per g wet weight of tissue (for the brain and duodenum samples) or as ng per mL (for the blood samples). The neurochemicals were determined with typical intra- and interassay coefficients of variation of less than 5 and 10%, respectively. The concentration of NMS in the various internal standard solutions and the volume of solution added to the different sample types were selected based on the typical sample weights and the expected range of analyte concentrations. Representative HPLC chromatograms of brain, duodenum, and blood samples are shown in Fig. 1.

Statistics

The statistical analysis was carried out using SPSS 11.5 (SPSS, Inc., Chicago, IL USA). All mean values are given as mean ± SEM. As clinical studies routinely report standard deviations, SD values are also given in Tables 1–3. All groups were tested for normality with the Shapiro–Wilk test. For normally distributed samples, means were compared using Student's two-tailed unpaired *t*-test (with Levene's test for the equality of variances) and cross-correlations were calculated using Pearson's coefficient of correlation. If either of the groups failed the normality test, the means were compared using the two-tailed Mann–Whitney test and cross-correlations were calculated using Spearman's coefficient of correlation. Two-tailed tests were used to test if correlation coefficients were different from zero. Every age group was treated as a separate experiment, i.e. no correction for the familywise error rate was used. No correction for the familywise error rate was used in multiple cross-correlations. In the linear regression analysis, the Shapiro–Wilk test was used to test the normality of residuals. In all tests, a *p*-value less than 0.05 was considered significant.

Results

Brain 5-HT

Three age groups (PD3, PD14, adult), each consisting of +/+ and –/– subgroups, were studied. At PD3, the mean fresh

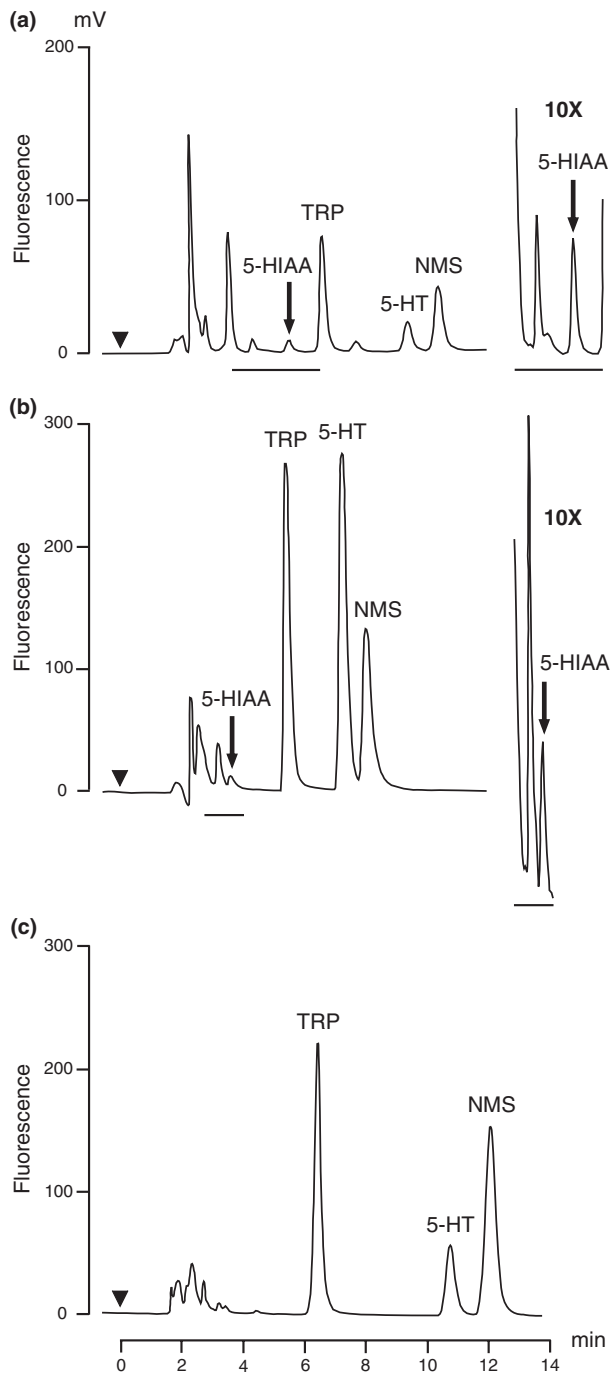


Fig. 1 Representative chromatograms of brain (a), duodenum (b), and blood (c) samples. The arrowheads mark the injection points. In (a) and (b), the underlined segments of the chromatograms are also shown at 10 × magnification. The scale of the y-axis (mV) applies to the unmagnified chromatograms. The internal standard (NMS) was added to the brain, gut and blood samples as a constituent of the homogenizing ISS at concentrations of 50, 114 and 323 ng/mL, as specified in the Materials and methods. Relative heights of the NMS peaks seen across samples are proportional to the NMS concentration in the homogenizing solution and the volume of the brain, gut and blood samples injected (10, 10, and 5 μ L, respectively).

weight of the whole $-/-$ brains was not different from the $+/+$ brains [156 ± 4 mg vs. 152 ± 4 mg respectively, $p > 0.2$ (Mann–Whitney test)]. In the adult $-/-$ mice, the overall distribution of neurons and SERT-positive fibers in the brain, as well as the distribution of serotonergic neurons in the dorsal raphe nucleus, appeared to be normal (Fig. 2). The means, SEM, and SD of the brain 5-HT levels (ng/g wet weight) in all groups are given in Table 1. The individual values of brain 5-HT levels are shown in Fig. 3(a). At PD3, the mean 5-HT levels in the $-/-$ group were 8% lower than the mean levels in the $+/+$ group, and this difference was significant ($p = 0.032$). No significant differences were found between the mean brain 5-HT levels of the $+/+$ and $-/-$ mice in the PD14 ($p > 0.2$) and adult ($p > 0.9$) groups.

Blood 5-HT

Three age groups (PD3, PD14, adult), each consisting of $+/+$ and $-/-$ subgroups, were studied. The platelet counts in five of the six groups were distributed normally ($p > 0.1$) and the distribution of the platelet counts in the remaining group (PD3, $-/-$) approached normality ($p = 0.042$). The mean platelet counts in the $+/+$ and $-/-$ groups were not significantly different at the three ages studied (in million platelets per milliliter, respectively: PD3 670 ± 61 vs. 551 ± 54 , $p > 0.1$ (*t*-test and the Mann–Whitney test); PD14 731 ± 20 vs. 802 ± 27 , $p > 0.05$; adult 834 ± 75 vs. 743 ± 39 , $p > 0.3$).

The blood 5-HT levels (ng/mL) in all six groups were normally distributed ($p > 0.10$). The means, SEM, and SD of the blood 5-HT levels in all groups are given in Table 3. The individual values of blood 5-HT levels are shown in Fig. 3(b). At PD3, the mean 5-HT levels of the $+/+$ and $-/-$ groups were not significantly different ($p > 0.5$). At PD14, the mean blood 5-HT levels of the $-/-$ mice were 24% higher than the mean levels of the $+/+$ mice, and this difference was highly significant ($p < 0.001$). The adult $-/-$ mice also had higher mean blood 5-HT levels than the adult $+/+$ mice, but this difference was not significant ($p > 0.2$).

Tryptophan, 5-hydroxyindoleacetic acid (5-HIAA) and 5-HIAA/5-HT levels

In order to gain insight into the possible causes of the altered 5-HT levels in the brain (PD3) and the blood (PD14) of the $-/-$ mice, we analyzed tryptophan and 5-HIAA levels in their brain and duodenum, as well as tryptophan levels in their blood. Also, the 5-HT turnover rates, represented as the ratios between the tissue 5-HIAA levels (ng/g) and the tissue 5-HT levels (ng/g), 5-HIAA/5-HT, were calculated for the brain and duodenum of each mouse. These results are presented in Tables 1–3.

At PD3, the $-/-$ mice had significantly lower tryptophan levels in the brain (29% reduction, $p = 0.006$), duodenum (26% reduction, $p = 0.009$), and blood (30% reduction, $p = 0.001$). The means, SEM and SD of the PD3 and PD14 mice

Table 1 Brain 5-HT, 5-HIAA, 5-HIAA/5-HT, and tryptophan levels in the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice

Substance	Age	Genotype	<i>n</i>	Mean	SEM	SD	<i>p</i>
5-HT, ng/g	PD3	+/+	18	198	6	26	0.032*
	PD3	-/-	19	182	4	18	
	PD14	+/+	12	204	8	27	0.231
	PD14	-/-	12	194	3	9	
	Adult ^a	+/+	5	564	10	22	0.917
	Adult	-/-	5	570	4	9	(MWT)
5-HIAA, ng/g	PD3	+/+	18	271	19	79	0.059
	PD3	-/-	19	228	12	54	
	PD14 ^a	+/+	12	150	5	18	0.214
	PD14	-/-	12	164	6	21	(MWT)
5-HIAA/5-HT	PD3	+/+	18	1.361	0.076	0.324	0.250
	PD3	-/-	19	1.250	0.058	0.253	
	PD14	+/+	12	0.738	0.012	0.042	0.002**
	PD14	-/-	12	0.847	0.026	0.089	
Tryptophan, ng/g	PD3	+/+	18	9009	767	3255	0.006**
	PD3	-/-	19	6387	446	1946	
	PD14	+/+	12	4819	220	763	0.511
	PD14	-/-	12	4651	116	404	

^aNon-normally distributed samples. Significant mean differences are indicated (**p* < 0.05, ***p* < 0.01). MWT, the Mann–Whitney test; *n*, number of cases.

Table 2 Duodenum 5-HT, 5-HIAA, 5-HIAA/5-HT, and tryptophan levels in the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice at PD3 and PD14

Substance	Age	Genotype	<i>n</i>	Mean	SEM	SD	<i>p</i>
5-HT, ng/g	PD3	+/+	18	3704	127	539	0.665
	PD3	-/-	18	3626	128	542	
	PD14	+/+	12	6328	331	1146	0.520
	PD14	-/-	12	6574	175	605	
5-HIAA, ng/g	PD3	+/+	18	433	29	122	0.246
	PD3	-/-	18	384	29	125	
	PD14 ^a	+/+	12	391	43	150	0.862
	PD14	-/-	12	388	50	172	(MWT)
5-HIAA/5-HT	PD3	+/+	18	0.118	0.008	0.033	0.201
	PD3	-/-	18	0.105	0.006	0.026	
	PD14	+/+	12	0.062	0.006	0.020	0.785
	PD14	-/-	12	0.059	0.008	0.028	
Tryptophan, ng/g	PD3	+/+	18	16 518	1019	4322	0.009**
	PD3	-/-	18	12 265	1134	4813	
	PD14	+/+	12	15 396	829	2870	0.316
	PD14	-/-	12	16 672	928	3216	

^aA non-normally distributed sample. **A highly significant mean difference is indicated. MWT, the Mann–Whitney test; *n*, number of cases.

are given in Tables 1–3, and the individual values of tryptophan levels are shown in Fig. 4.

At PD14, the -/- mice had significantly higher (15%) 5-HT turnover rates in the brain compared with the +/+ mice (*p* = 0.002), and the blood tryptophan levels of the -/- mice were significantly higher (21%) compared with the +/+ mice (*p* = 0.003).

We proceeded to calculate the intra-animal correlations between the 5-HT, 5-HIAA, 5-HIAA/5-HT, and tryptophan levels in the three studied tissues (Fig. 5). At PD14, the duodenum 5-HIAA levels and 5-HIAA/5-HT ratios correlated with the brain 5-HT levels of the +/+ mice; and, in the -/- mice, the duodenum 5-HIAA levels and 5-HIAA/5-HT ratios correlated with the blood 5-HT levels. In order to explore

Substance	Age	Genotype	<i>n</i>	Mean	SEM	SD	<i>p</i>
5-HT, ng/mL	PD3	+/+	14	1318	90	338	0.551
	PD3	-/-	20	1402	99	441	
	PD14	+/+	10	2103	55	173	< 0.001**
	PD14	-/-	12	2616	65	226	
	Adult	+/+	6	3820	287	704	0.270
	Adult	-/-	5	4318	309	691	
Tryptophan, ng/mL	PD3	+/+	14	15 557	890	3330	0.001**
	PD3	-/-	20	10 839	873	3903	
	PD14	+/+	10	11 563	344	1087	0.003**
	PD14 ^a	-/-	12	13 936	764	2648	(MWT)

^aA non-normally distributed sample. **Highly significant mean differences are indicated. MWT, the Mann-Whitney test; *n*, number of cases.

Table 3 Blood 5-HT and tryptophan levels in the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice

these relationships further, we used linear regression. The duodenum 5-HIAA levels and 5-HIAA/5-HT ratios were used as independent variables and were entered using the forward and backward methods. The regression procedure determined that the optimal prediction was achieved by using the duodenum 5-HIAA/5-HT ratios only. The duodenum 5-HIAA/5-HT ratios accounted for 59% of the variance in the brain 5-HT levels of the +/+ mice ($R^2 = 0.594$, $F_{1,10} = 14.6$, $p = 0.003$) and for 50% of the variance in the blood 5-HT levels of the -/- mice ($R^2 = 0.501$, $F_{1,10} = 10.0$, $p = 0.010$). Stein's adjusted R^2 (Field 2005), which indicates how well a regression model cross-validates, was 0.462 and 0.339, respectively. In both cases, the standard assumptions about residuals were met: they were normally distributed ($p > 0.3$ and $p > 0.8$, respectively) and independent (the Durbin-Watson statistic was 1.91 and 1.99, respectively). The regression results are shown in Fig. 6(a and d). For comparison, linear regression analysis was also performed for the brain 5-HT levels of the -/- mice (PD14) and for the blood 5-HT levels of the +/+ mice (PD14). The duodenum 5-HIAA/5-HT ratio was used as the sole independent variable, which was force-entered. Its predictive value did not reach significance in these mice ($p > 0.1$; Figs 6b and c).

Discussion

The following are the main findings of the present study: (i) at 3 days after birth, the 5-HT_{1A}-deficient mice had significantly reduced (by 8%) brain 5-HT levels and normal blood 5-HT levels; (ii) at 2 weeks after birth, the 5-HT_{1A}-deficient mice had normal brain 5-HT levels, but their blood 5-HT levels were significantly higher than normal (by 24%); and (iii) in the adult 5-HT_{1A}-deficient mice, both the brain and blood 5-HT levels were not significantly different from those of the wild-type mice.

The 8% reduction in brain 5-HT levels at PD3 is unlikely to have a major effect on normal brain development or

function. For instance, even a 70% loss of serotonergic neurons in the brain of *Pet-1* -/- mice does not alter the gross cytoarchitecture of the brain (Hendricks *et al.* 2003). However, this finding raises the issue of whether 5-HT_{1A}-deficient mice may have still lower brain 5-HT levels before birth. Interestingly, the brain 5-HT levels of the 5-HT_{1A}-deficient mice appeared to normalize soon after birth: at PD14, they were no longer different from those of the wild-type mice; and, consistent with previous studies (Heisler *et al.* 1998), the adult 5-HT_{1A}-deficient and wild-type mice had the same mean brain 5-HT levels. The transiently low 5-HT levels in the brain of the 5-HT_{1A}-deficient mice could be caused by low tryptophan levels (Fig. 4), given the relationship between tryptophan levels and 5-HT synthesis *in vivo* (Young and Teff 1989) and the highly significant correlation between brain tryptophan and brain 5-HT levels in the wild-type and 5-HT_{1A}-deficient PD3 mice (Fig. 5). As tryptophan can cross the blood-brain barrier, it is not surprising that, in the 5-HT_{1A}-deficient PD3 mice, tryptophan levels in the brain, duodenum and blood were similarly reduced. However, it is unclear what caused these low tryptophan levels.

Other factors may influence the dynamics of 5-HT levels in the developing brain. In the rat hippocampus, the proportion of astrocytes that co-express S-100 β and 5-HT_{1A} receptors with respect to all S-100 β -positive astrocytes drops from more than 90% at PD7 to less than 25% at PD16 (Patel and Zhou 2005). During brain development, the stimulation of astrocytic 5-HT_{1A} receptors increases the release of S-100 β , which in turn stimulates the growth of serotonergic terminals (Whitaker-Azmitia and Azmitia 1994; Whitaker-Azmitia 2001). It is possible that soon after birth the development of serotonergic terminals becomes less dependent on 5-HT_{1A} receptors, and the brain 5-HT levels of 5-HT_{1A}-deficient mice catch up with the brain 5-HT levels of wild-type mice. Also, a recent study has shown that the C57BL/6 mouse strain transiently expresses tryptophan hydroxylase 1 (*Tph1*) in the brainstem after PD7 (Nakamura *et al.* 2006). While 5-HT_{1A} receptors can regulate the

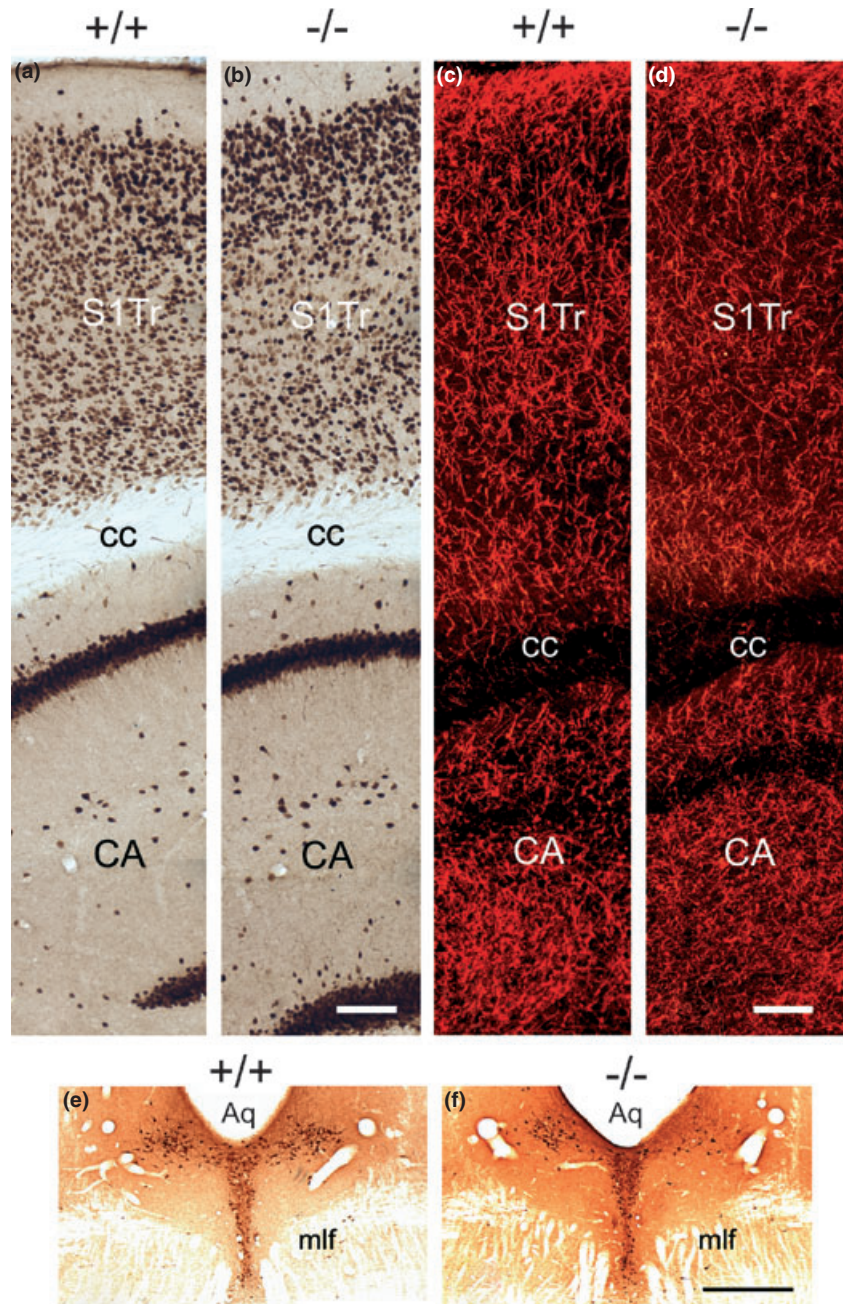


Fig. 2 The distribution of neurons and serotonergic fibers does not appear to be altered in adult mutant ($5\text{-HT}_{1A}^{-/-}$) mice, and their dorsal raphe nucleus also appears normal. (a, b) NeuN immunoreactivity in the somatosensory cortex and the hippocampus of adult $+/+$ and $-/-$ males; (c, d) SERT immunoreactivity in the somatosensory cortex and the hippocampus of adult $+/+$ and $-/-$ males; (e, f) 5-HT immunoreactivity in the dorsal raphe nucleus of adult $+/+$ and $-/-$ females. Aq, cerebral aqueduct; CA, hippocampus; cc, corpus callosum; mlf, medial longitudinal fasciculus; S1Tr, primary somatosensory cortex (trunk region). Scale bars, 100 μm (a–d) and 500 μm (e–f).

expression of tryptophan hydroxylase 2 (*Tph2*) (Remes Lenicov and Albert 2005), the 'main' tryptophan hydroxylase of the brain (Walther *et al.* 2003), no information is available on how these receptors may affect the transient post-natal expression of *Tph1*, which has higher affinity to tryptophan than *Tph2* and which may influence post-natal 5-HT levels in the brain (Nakamura *et al.* 2006).

At PD14, the 5-HT turnover (as measured by the 5-HIAA/5-HT ratio) in the frontal brain of the 5-HT_{1A} -deficient mice was 15% higher compared with the wild-type mice, even although the 5-HT levels in the same

tissue were not different from those of wild-type mice (Table 1). A remarkably similar increase in 5-HT turnover has been observed in the striatal tissue of mice lacking p11, a protein that increases membrane localization of 5-HT_{1B} receptors (Svenningsson *et al.* 2006). Whether this similarity between $5\text{-HT}_{1A}^{-/-}$ and p11 $-/-$ mice is coincidental or indicates a common underlying mechanism remains to be investigated.

By 2 weeks after birth, the 5-HT_{1A} -deficient mice had developed platelet hyperserotonemia. Interestingly, the relative elevation (24%) of their mean 5-HT levels in the blood

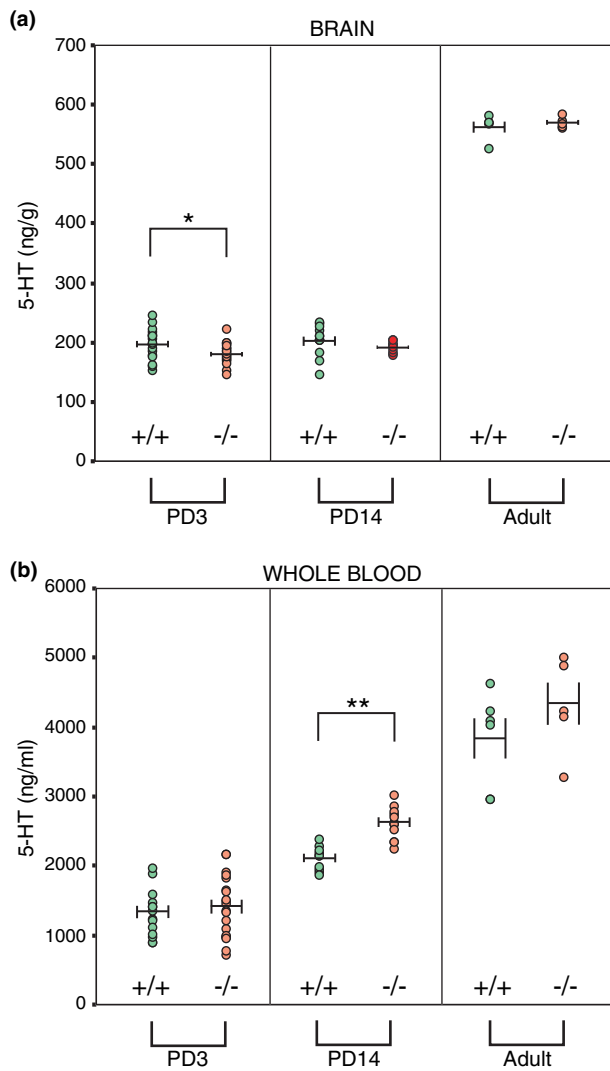


Fig. 3 5-HT levels in the brain (a) and the whole blood (b) of the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice post-natally (PD3, PD14) and in adulthood. The circles represent individual animals (some circles overlap), the horizontal bars are the means, and the error bars are the SEMs. The brain 5-HT levels were measured in the whole brains of the PD3 mice and in the frontal brains of the PD14 and adult mice. At PD3, the -/- mice had significantly lower brain 5-HT levels compared with the +/+ mice (**p* < 0.05). At PD14, the -/- mice had significantly higher blood 5-HT levels compared with the +/+ mice (***p* < 0.001). The other differences between +/+ and -/- mice were not statistically significant.

was similar to what is typically observed in autistic groups (Anderson *et al.* 1990; Mulder *et al.* 2004). In the 5-HT_{1A}-deficient mice, this phenomenon may have been caused by altered 5-HT release from gut EC cells that normally express 5-HT_{1A} receptors (Kirchgeßner *et al.* 1996) and that synthesize virtually all 5-HT found in blood platelets. The mechanism of this proposed increase in gut 5-HT release remains unclear, because the function of 5-HT_{1A} receptors in EC cells is not well understood. In the brain, 5-HT_{1A} receptors

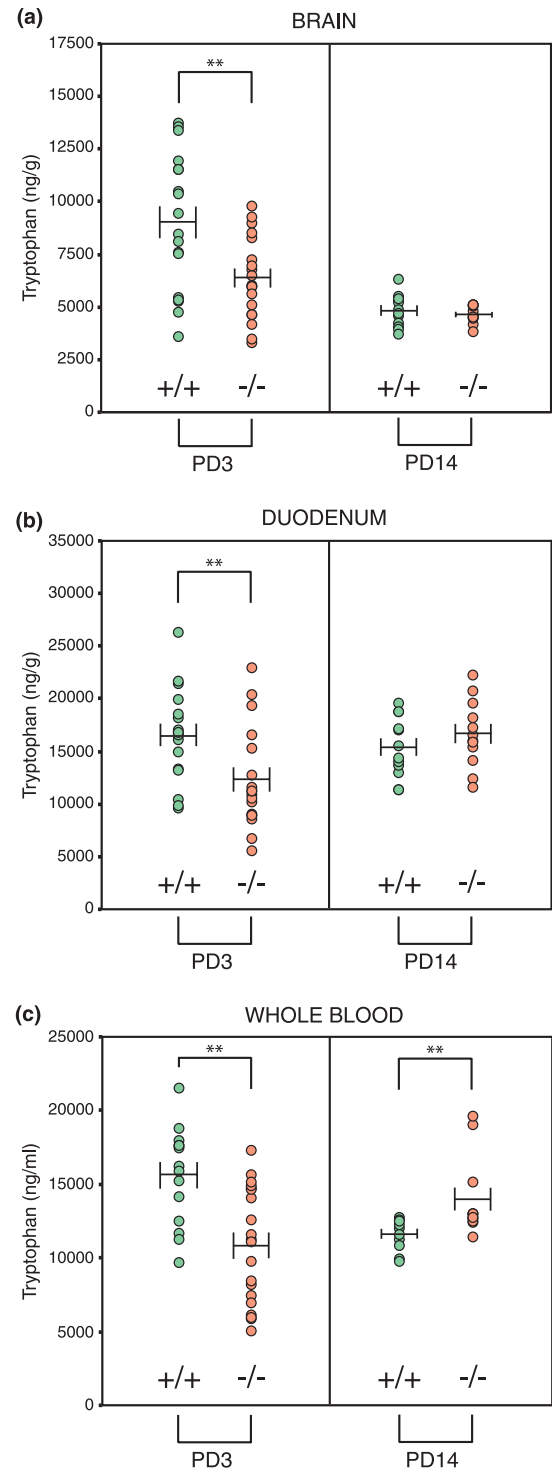


Fig. 4 Tryptophan levels in the brain (a), the duodenum (b), and the whole blood (c) of the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice at PD3 and PD14. The circles represent individual animals (some circles overlap), the horizontal bars are the means, and the error bars are the SEM. The brain tryptophan levels were measured in the whole brains of the PD3 mice and in the frontal brains of the PD14 mice. At PD3, the -/- mice had significantly lower tryptophan levels in all three tissues (***p* < 0.01).

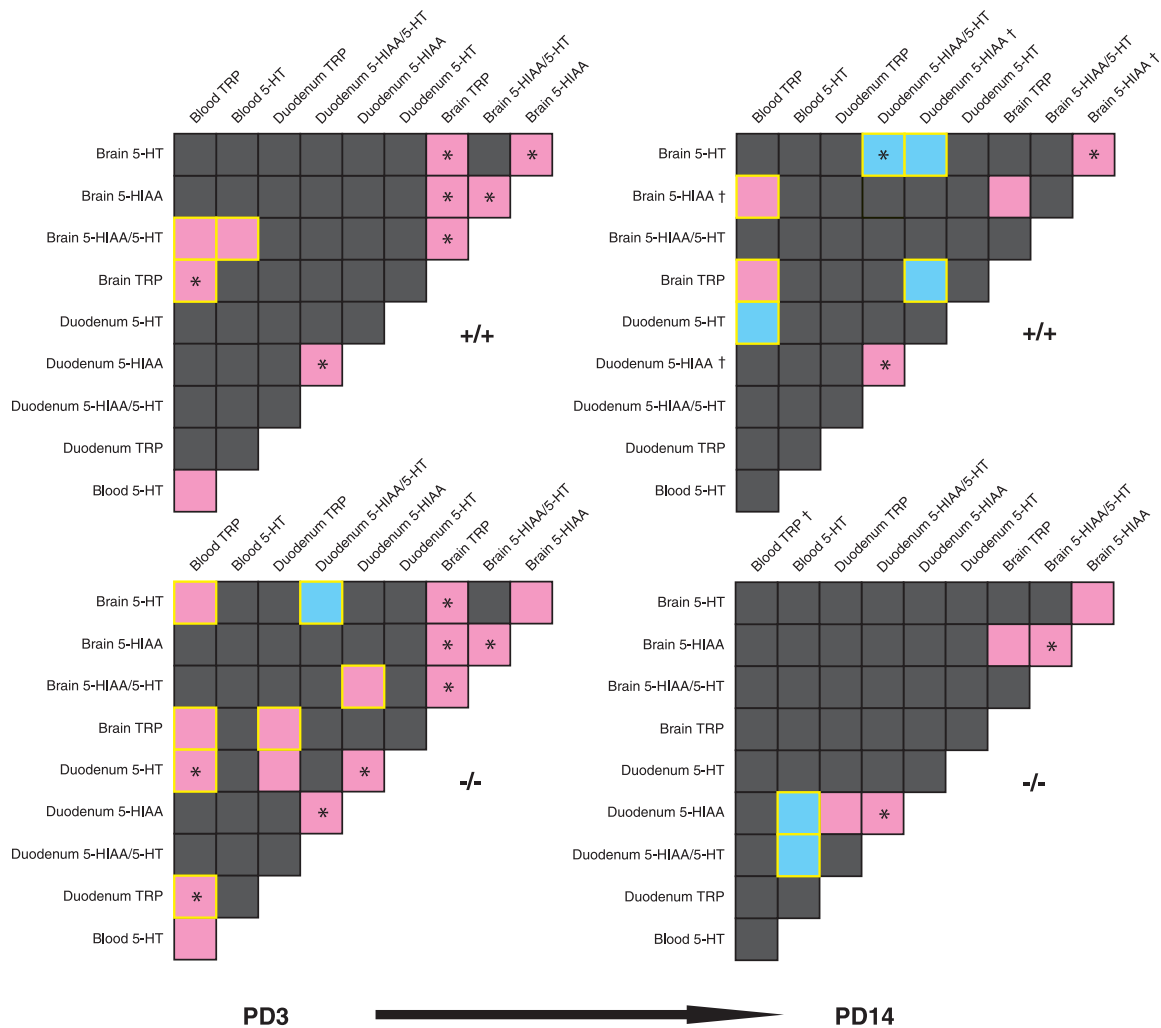


Fig. 5 Cross-correlations between 5-HT, 5-HIAA, 5-HIAA/5-HT, and tryptophan levels in the brain, the duodenum, and the blood of the wild-type (+/+) and mutant (5-HT_{1A} -/-) mice at PD3 and PD14. For normally distributed samples, Pearson's correlation coefficient was used. If either of the samples was not distributed normally (†), Spearman's correlation coefficient was used instead. Correlation coefficients not significantly different from zero are shown in grey; positive correlation coefficients significantly different from zero are shown in purple; and negative correlation coefficients significantly different from zero are shown in blue. Correlation coefficients highly

significantly different from zero ($p < 0.01$) are marked with an asterisk. Significant cross-system (brain vs. duodenum, duodenum vs. blood, brain vs. blood) correlations are boxed in yellow. It should be noted that (i) correlations do not imply causality; (ii) generally, multiple cross-correlations are not independent; (iii) the theoretical maximal absolute value of a correlation coefficient is limited by the measurement error (the attenuation of the correlation coefficient). For the purpose of this analysis, HPLC measurement errors can be considered to be negligible (less than 10%).

expressed in serotonergic neurons can regulate 5-HT release from these neurons (Adell *et al.* 2002), suggesting that 5-HT_{1A} receptors might serve a similar function in EC cells. However, 5-HT_{1A} receptors may be coupled to a number of G-proteins and may be functionally different in different areas of the brain (Lanfumeu and Hamon 2004), so caution should be exercised in drawing such parallels.

It appears less likely that the observed blood hyperserotonemia at PD14 could be caused by altered 5-HT uptake by blood platelets, because platelets are not known to express

5-HT_{1A} receptors. It is also unlikely that it was caused by altered precursor availability, because the duodenum tryptophan levels were normal (Table 2). Also, the correlation between blood tryptophan and blood 5-HT levels was not significant in PD14 mice (Fig. 5), which is consistent with observations in humans, both normal and autistic (Minderaa *et al.* 1987).

Blood 5-HT levels may also depend on how much 5-HT is removed from the circulation after the gut releases it into the blood. A large proportion of 5-HT is cleared by the

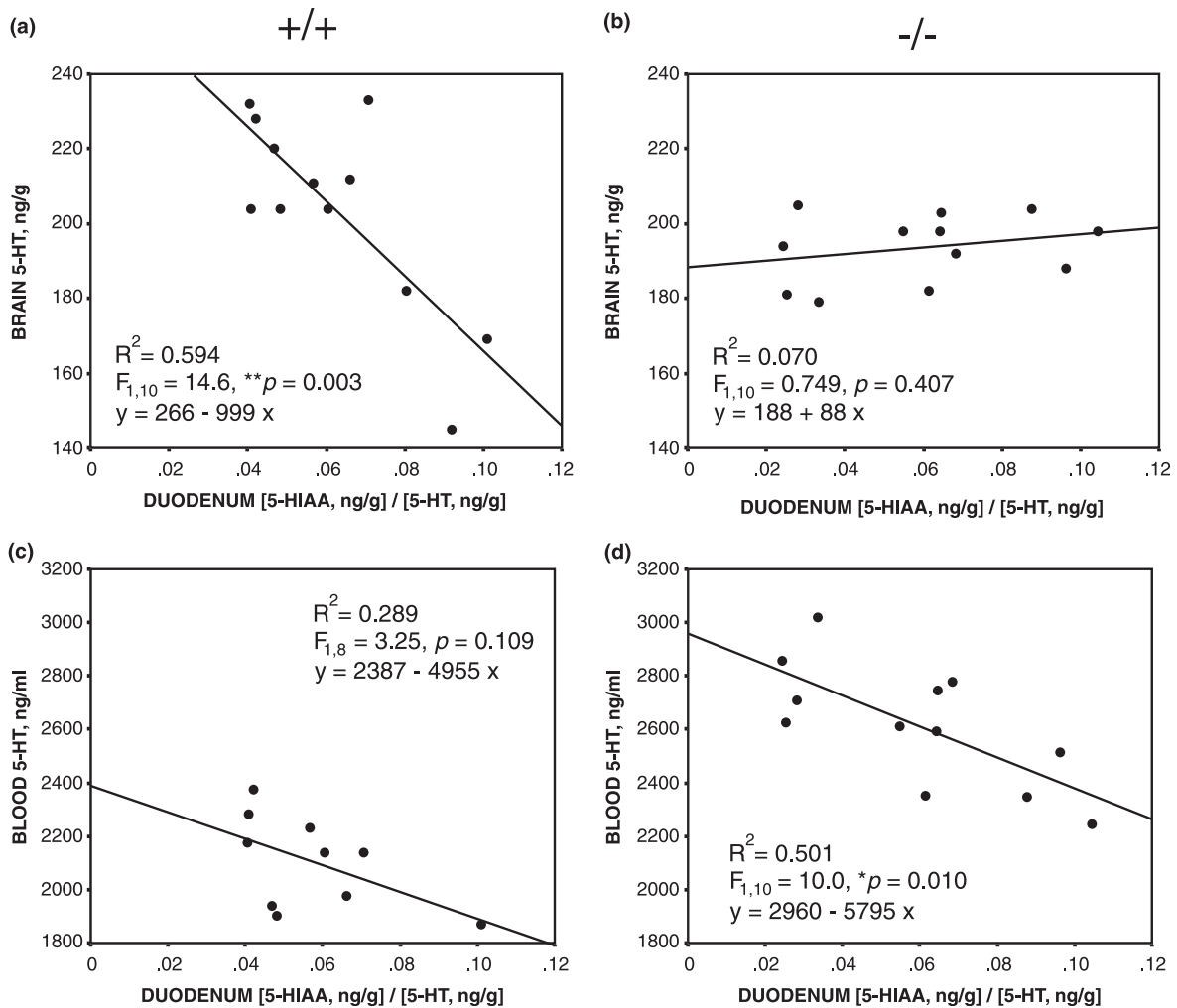


Fig. 6 Linear regression analysis of how well the duodenum 5-HIAA/5-HT ratios predicted the 5-HT levels in the brain (a, b) and blood (c, d) of the PD14 wild-type (+/+) and mutant (5-HT_{1A} -/-) mice. The 5-HIAA/5-HT ratios well predicted the brain 5-HT levels in the wild-type

mice and the blood 5-HT levels in the mutant mice. The intercept and slope in (a) are significantly different from those in (b) ($p < 0.001$). The intercept in (c) is significantly different from that in (d) ($p = 0.013$), but the slopes in (c) and (d) are not significantly different ($p = 0.806$).

liver and lungs (Thomas and Vane 1967; Anderson *et al.* 1987). However, the expression of 5-HT_{1A} receptors in the liver appears negligible (Zhou *et al.* 1999). Also, 5-HT_{1A} receptors do not mediate 5-HT-induced pulmonary responses in C57BL/6 mice (Martin *et al.* 1994) and are not expressed in human intrapulmonary arteries and veins (Morcillo and Cortijo 1999). Notably, the kidneys contribute to 5-HT clearance from the blood circulation (Anderson *et al.* 1987) and they do express 5-HT_{1A} receptors in humans and rats (Raymond *et al.* 1993; Zhou *et al.* 1999), so their role in the blood hyperserotonemia of the 5-HT_{1A}-deficient mice cannot be ruled out.

It is possible that the absence of functional 5-HT_{1A} receptors could alter the expression of other genes that affect blood 5-HT levels. For instance, the absence of 5-HT_{1A} receptors from the gut might lead to over-expression of *Tph1*,

which synthesizes virtually all 5-HT found in the blood (Walther *et al.* 2003). In the brain, 5-HT_{1A} receptors have been recently shown to regulate the expression of *Tph2* (Remes Lenicov and Albert 2005).

Interestingly, the duodenum 5-HT turnover rates (5-HIAA/5-HT) well predicted the brain 5-HT levels of the PD14 wild-type mice (Fig. 6a). This relationship may reflect parallel processes involving 5-HT_{1A} receptors in the gut and the brain. Consistent with this hypothesis, this relationship was completely lost in the 5-HT_{1A}-deficient mice (Fig. 6b). Instead, in the mutant mice, the duodenum 5-HT turnover rates well predicted the blood 5-HT levels (Fig. 6d). The negative linear relationship between these variables is somewhat counterintuitive but expected on theoretical grounds, if blood 5-HT levels are controlled by negative feedback (Janušonis 2005). The potential

relevance of these findings to human studies awaits further research.

The present study may provide new insights into the developmental neurobiology of autism. Our results suggest that the hyperserotonemia of autism may be preceded by low 5-HT levels in the developing brain, at which time platelet 5-HT levels may be still normal. In the 5-HT_{1A}-deficient mice, the platelet hyperserotonemia appeared to develop in the first two post-natal weeks. However, the mouse neocortex continues to generate new neurons up until birth (Polleux *et al.* 1997), whereas most neurons in the human neocortex are generated by the mid-gestation (Rakic 2000). Also, the first serotonergic fibers in the mouse neocortex appear around embryonic day 16 (Bruning *et al.* 1997), a few days before birth, whereas in the human neocortex they are detected as early as gestational week 13 (Verney *et al.* 2002). While less is known about the development of EC cells in the mouse and human gut, these species differences suggest that the platelet hyperserotonemia of autism might develop before birth.

In the 5-HT_{1A}-deficient mice, the low brain 5-HT levels at PD3 could be caused by low central and peripheral tryptophan levels. While the total blood tryptophan levels do not appear to be significantly altered in autism (Minderaa *et al.* 1987; D'Eufemia *et al.* 1995), the proportion of tryptophan with respect to other large neutral amino acids (transported into the brain by the same carrier) may be in fact be lower than normal in autism (D'Eufemia *et al.* 1995). It also remains unclear if the synthesis of 5-HT in the gut of hyperserotonemic individuals is increased (Minderaa *et al.* 1987; Mulder *et al.* 2004; Croonenberghs *et al.* 2005). Further research in this area appears necessary, with careful study of 5-HIAA urine excretion rates and other potential indices of gut 5-HT production warranted.

It should be noted that we are not suggesting that autistic individuals have non-functional 5-HT_{1A} receptors. Although these receptors have been hypothesized to be involved in autism (Chugani 2002), their exact role remains unclear. By using mice lacking functional 5-HT_{1A} receptors, we have shown, for the first time, that altered expression of a gene normally expressed in both the developing brain and the gut (i) may cause behavioral abnormalities (Heisler *et al.* 1998) with no alterations of gross brain anatomy and (ii) may cause platelet hyperserotonemia. We suggest that genes with functions in both brain and gut serotonergic biology should be carefully studied for their possible role in the hyperserotonemia of autism and in the underlying neurobiology of autistic behavior.

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