LETTER

doi:10.1038/nature09972

A transient placental source of serotonin for the fetal forebrain

Alexandre Bonnin[1,2], Nick Goeden[1], Kevin Chen[3], Melissa L. Wilson[4], Jennifer King[4], Jean C. Shih[3], Randy D. Blakely[5,6], Evan S. Deneris[7] & Pat Levitt[1,2]

Serotonin (5-hydroxytryptamine or 5-HT) is thought to regulate neurodevelopmental processes through maternal–fetal interactions that have long-term mental health implications. It is thought that beyond fetal 5-HT neurons there are significant maternal contributions to fetal 5-HT during pregnancy[1,2] but this has not been tested empirically. To examine putative central and peripheral sources of embryonic brain 5-HT, we used *Pet1*$^{-/-}$ (also called Fev) mice in which most dorsal raphe neurons lack 5-HT[3]. We detected previously unknown differences in accumulation of 5-HT between the forebrain and hindbrain during early and late fetal stages, through an exogenous source of 5-HT which is not of maternal origin. Using additional genetic strategies, a new technology for studying placental biology *ex vivo* and direct manipulation of placental neosynthesis, we investigated the nature of this exogenous source. We uncovered a placental 5-HT synthetic pathway from a maternal tryptophan precursor in both mice and humans. This study reveals a new, direct role for placental metabolic pathways in modulating fetal brain development and indicates that maternal–placental–fetal interactions could underlie the pronounced impact of 5-HT on long-lasting mental health outcomes.

Fetal 5-HT dysfunction is implicated in developmental programming by altering brain circuit formation[4], which later translates into abnormal adult behaviours[5,6]. In humans, risk alleles in genes involved in 5-HT function combine with early adverse experiences during development to affect adult-onset mental illnesses[7]. Furthermore, polymorphisms in the 5-HT transporter (*SLC6A4*, which encodes SERT) and 5-HT receptors, which are expressed early in brain development[8], are associated with neurodevelopmental disorders such as autism spectrum disorder and schizophrenia[9,10]. A puzzling issue regarding the role of 5-HT in fetal brain development is that receptors, transporters and degrading enzymes for 5-HT often appear before the development of 5-HT innervation[11] itself, suggesting the existence of an exogenous source of 5-HT at early stages of development. The most biologically influential source of exogenous 5-HT is claimed to be maternal[1,2] but there is a lack of experimental data to support such a mechanism of developmental programming.

Analysis of fetal *Pet1*$^{-/-}$ mice provides an opportunity to assess potential extra-embryonic sources of 5-HT, as only ~30% of dorsal raphe 5-HT neurons can be detected in null mice, which also express low levels of tryptophan hydrolase 2 (TPH2), aromatic l-amino acid decarboxylase (AADC; also called dopa decarboxylase, Ddc), SERT and other markers of the serotonergic phenotype[3]. To assess whether dorsal raphe neurons are also the sole source of brain 5-HT during development, we compared the concentration of 5-HT in embryonic brains collected from *Pet1*$^{-/-}$ and wild-type littermates from embryonic day (E) 10.5, the onset of 5-HT synthesis in dorsal raphe neurons, to E17.5, when 5-HT axons are fully deployed throughout the forebrain. High-performance liquid chromatography (HPLC) was used to measure the concentration of 5-HT in the mid/hindbrain region (termed 'hindbrain'), which contains 5-HT cell bodies and proximal axons, and in the forebrain, which contains only distal 5-HT axons[12,13]. Consistent with dorsal raphe neurons providing the main source of 5-HT in the hindbrain, the 5-HT concentration was lower in *Pet1*$^{-/-}$ hindbrains than in those of wild-type mice at every age tested (Fig. 1a). Notably, in the *Pet1*$^{-/-}$ forebrain, 5-HT levels were statistically indistinguishable from wild type at E10.5 to E15.5; however, large differences emerged at E16.5 (Fig. 1b). This is consistent with dorsal raphe axons being the major source of forebrain 5-HT at this and later ages but not earlier (Supplementary Fig. 2 and ref. 3). Remarkably, even before the arrival of 5-HT axons in the ventral forebrain (E10.5–E12.5), low levels of 5-HT were detected (Fig. 1b). Normally, over the next three embryonic days, progressively more 5-HT axons grow into the forebrain[13] (Fig. 1c–e). In the *Pet1*$^{-/-}$ forebrain, however, there is a marked reduction in 5-HT axon density compared to wild type (Fig. 1c–i), even though total tissue 5-HT concentrations are comparable. The density and distribution of thalamocortical axons, which also express SERT and can take up 5-HT[14], are similar in *Pet1*$^{-/-}$ and wild-type mice (Fig. 1c, f). These results reveal a complex regulation of 5-HT in the fetal brain, with dorsal raphe serotonergic neurons and axons representing the major source of 5-HT in the hindbrain and at later embryonic stages in the forebrain but not the main source of 5-HT in the early developing forebrain.

The greater decrease in total tissue 5-HT concentration in the hindbrain than in the forebrain in *Pet1*$^{-/-}$ mice suggests a differential contribution of non-dorsal-raphe sources in these regions. Alternatively, because 5-HT degradation enzyme (monoamine oxydase A; MAO-A) activity is higher in the hindbrain than in the forebrain at early stages of development[15], a differential degradation of 5-HT across the two brain regions may account for the difference. Consistent with this latter possibility, 5-hydroxyindoleacetic acid (5-HIAA) concentration in the E14.5 s*Maoa*$^{-/-}$ mouse (which lacks MAOA enzymatic activity and cannot efficiently degrade 5-HT[16]) were decreased 3.4-fold in the forebrain, but 6.1-fold in the hindbrain compared to wild type littermates (Supplementary Fig. 3). In contrast, at E16.5, 5-HIAA concentrations were decreased to a similar extent in the s*Maoa*$^{-/-}$ forebrain and hindbrain (3.6- and 3.1-fold respectively). A downregulation of MAOA activity in the *Pet1*$^{-/-}$ forebrain before E16.5 could explain the normal concentrations of 5-HT measured in the region. To test this possibility, we quantified MAOA activity in the forebrain at E14.5 and showed that it was not different in *Pet1*$^{-/-}$, *Pet1*$^{+/-}$ and *Pet1*$^{+/+}$ forebrains (Supplementary Table 2). Furthermore, 5-HIAA concentrations were not different in the *Pet1*$^{-/-}$ and *Pet1*$^{+/+}$ forebrains at E12.5 and E14.5 (Supplementary Fig. 3c). The data demonstrate that MAO-A activity is not downregulated in the *Pet1*$^{-/-}$ forebrain.

The non-dorsal-raphe origin of 5-HT in the early fetal forebrain could arise from several sources. Because adult forebrain catecholaminergic

[1]Zilkha Neurogenetic Institute, Keck School of Medicine of USC, Los Angeles, California 90089, USA. [2]Department of Cell & Neurobiology, Keck School of Medicine of USC, Los Angeles, California 90089, USA. [3]Department of Pharmacology & Pharmaceutical Sciences, USC School of Pharmacy, Los Angeles, California 90089, USA. [4]Departments of OB/GYN & Preventive Medicine, Keck School of Medicine of USC, Los Angeles, California 90089, USA. [5]Department of Pharmacology, Vanderbilt University Medical Center, Nashville, Tennessee 37221, USA. [6]Center for Molecular Neuroscience, Vanderbilt University Medical Center, Nashville, Tennessee 37221, USA. [7]Department of Neuroscience, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, USA.

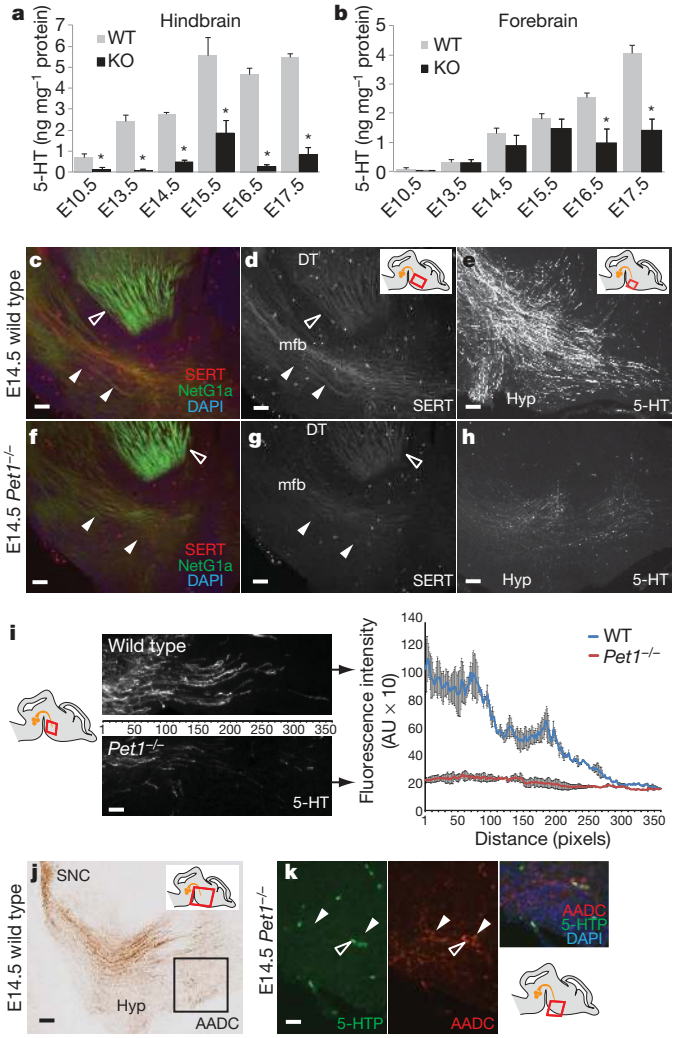

Figure 1 | Comparison of fetal 5-HT concentrations in the hindbrain and forebrain of *Pet1*$^{-/-}$ and wild type embryos from E10.5 to E17.5. **a**, 5-HT concentration in the *Pet1*$^{-/-}$ (KO) hindbrain is significantly lower than in wild-type (WT) littermates at every age tested. **b**, In contrast, 5-HT concentrations in the *Pet1*$^{-/-}$ forebrain are not significantly different from wild-type littermates from E10.5 to E15.5 but become significantly lower at E16.5 and E17.5 ($n \geq 6$ embryos per genotype per age; *, $P < 0.005$; one-way analysis of variance (ANOVA); data are presented as means ± s.e.m.). **c–h**, Serotonergic axons (SERT^{+}) and dorsal thalamic (DT) axons (NetG1a^{+}) immunostained on sagittal sections at E14.5 in wild-type (**c**) and *Pet1*$^{-/-}$ (**f**) embryos (regions shown correspond to the red box in the drawings). In wild-type E14.5 embryos (**d**) SERT^{+} axons grow ventrally into the forebrain through the medial forebrain bundle (mfb, white arrowheads); SERT also labels dorsal thalamic axons at this age (open arrowhead). In comparison, very few SERT^{+} axons remain in the *Pet1*$^{-/-}$ (**g**). The pattern and density of SERT^{+} DT axons appear to be unaffected in the *Pet1*$^{-/-}$ forebrain. Scale bars: 50 µm. The rostral-most extent of ingrowing serotonergic axons immunolabelled with 5-HT in the wild type (**e**) shows numerous 5-HT^{+} axons, some of which diverge towards the hypothalamus (Hyp). In contrast, only few 5-HT^{+} axons remain in the *Pet1*$^{-/-}$ forebrain (**h**). Scale bars: 20 µm. **i**, Densitometric analysis of 5-HT^{+} axons in the most rostral part of the medial forebrain bundle (region indicated in the diagram) at E14.5 confirms fewer axons in the *Pet1*$^{-/-}$. AU, arbitrary units. **j**, AADC staining identifies dopaminergic neurons in the substantia nigra pars compacta (SNC), along with dopaminergic and serotonergic axons coursing through the ventral forebrain at E14.5, and also AADC^{+} catecholaminergic neurons present in the hypothalamus (Hyp, black box). Scale bar: 100 µm. **k**, AADC^{+} neurons in the hypothalamic region (red box in bottom right drawing; middle panel, white arrowheads) are 5-HTP-negative. Open arrowheads indicate fluorescence from blood vessels. Scale bar: 25 µm.

and dopaminergic neurons express the AADC enzyme, these cells can ectopically synthesize 5-HT, albeit after administration of large doses of the precursor 5-HTP[17]. We tested the possibility that embryonic AADC-expressing neurons could ectopically produce 5-HT in the early *Pet1*$^{-/-}$ forebrain. Consistent with measures of unaltered dopamine concentration (Supplementary Fig. 1), AADC immunostaining revealed normal catecholaminergic and dopaminergic neuron and axon density in the *Pet1*$^{-/-}$ forebrain (Fig. 1j). Furthermore, catecholaminergic neurons present in the *Pet1*$^{-/-}$ hypothalamus do not exhibit ectopic 5-HTP or 5-HT immunoreactivity (Fig. 1k), consistent with there being no local cellular source of 5-HT in the *Pet1*$^{-/-}$ forebrain.

In the developing forebrain, E10.5–E15.5 is the period of pronounced neurogenesis and axon growth. As 5-HT modulates both processes[4,10], it is essential that its availability be regulated during this time. It is remarkable, therefore, that over this time period, even in the absence of 5-HT axons, the concentration of 5-HT in the *Pet1*$^{-/-}$ forebrain is normal. Possible exogenous sources include the embryonic gut, the maternal blood through the placenta, or the placenta itself. We ruled out the embryonic gut as a source because expression of the 5-HT biosynthetic enzyme tryptophan hydroxylase (TPH1), which provides blood 5-HT, begins late (E15.5) in fetal enterochromaffin cells[1,18]. To test the possibility that maternal 5-HT is transferred to the fetal brain, we examined brains from fetuses of *Sert* knockout (*Sert*$^{-/-}$) dams; total blood and platelets in these dams contain virtually no 5-HT[19]. This absence of blood 5-HT is attributed to a failure of uptake by platelets, to rapid degradation of the remaining free plasma 5-HT in the liver and to compensatory uptake by other transporters in the gut[19]. Despite this, the concentration of 5-HT is not different in the forebrain of *Sert*$^{+/-}$ E12.5 embryos from *Sert*$^{-/-}$ or wild-type dams (Supplementary Table 1), indicating that maternal blood 5-HT is not the main source of fetal blood and forebrain 5-HT at early stages of development.

We considered the alternative hypothesis that the essential amino acid tryptophan, originating from the pregnant dam, would be converted to 5-HT in the placenta and delivered to the fetal circulation. Injection of tryptophan in pregnant dams increases 5-HT concentration in the fetal brain[20] but the precise location of the synthetic conversion has never been identified. Quantitative RT-PCR of placental tissue detected transcripts of *Tph1* and *Aadc*, but not *Pet1* (Supplementary Fig. 4a). Immunocytochemistry confirmed that TPH1 and AADC proteins are expressed in the syncytiotrophoblastic cell layer of the placenta at E10.5–E14.5 (Fig. 2c–h and Supplementary Fig. 4e–j). The placenta thus has the necessary machinery to synthesize 5-HT. We tested for placental 5-HT neosynthesis and showed that both 5-HTP and 5-HT neosyntheses occur in placenta and fetal hindbrain extracts incubated with tryptophan (Fig. 2a) as early as E10.5 (Supplementary Fig. 4b). Placental 5-HT synthesis capacity was greater at E14.5 than at E18.5, whereas the converse was true in the hindbrain, consistent with changes in *Tph1* transcript expression (data not shown). The capacity for placental 5-HT synthesis at E14.5 was not affected by the absence of embryonic *Pet1* gene expression (Supplementary Fig. 4c). This synthetic capacity is not unique to mice, as human placental fetal villi at 11 weeks of gestation showed robust 5-HT neo-synthesis (Fig. 2b), indicating that a placental source of 5-HT is important for human fetal development.

Although not providing conclusive proof, these data are consistent with the possibility that neosynthesis and transport of 5-HT from a maternal tryptophan precursor occurs in an intact placenta. We addressed this with two strategies. First, we developed a novel *ex vivo* technology for regulating placental organ perfusion, thus allowing the presentation of maternal precursor and the collection of fetal perfusate in intact, live murine placentas (Fig. 2i). Within 15 min of tryptophan injection through the maternal uterine artery, there was a large accumulation of newly synthesized 5-HT that passes through the fetal placental circulation (Fig. 2j), demonstrating that the live placenta is able to convert tryptophan to 5-HT and release the neurotransmitter into the fetal circulation. In contrast, when 5-HT (1.5 nM) was injected into the uterine artery, only 0.32 ± 0.16% of the maternal free 5-HT was transferred to the fetal umbilical vein during a 30 min perfusion

period. In a second strategy, we specifically blocked placental TPH1 enzyme activity by microinjecting small volumes of the TPH inhibitor p-chlorophenyalanine (PCPA) directly into the labyrinth zone of E14.5 placentas *in utero* (Fig. 3a). To minimize nonspecific effects due to diffusion of the drug into the maternal and fetal blood compartments, placental and fetal brain tissues were collected after a short 30-min period of drug exposure. This pharmacological manipulation reduced 5-HT levels in the placenta but did not reduce 5-HT in the fetal hindbrain, indicating that exposure to PCPA was too short to inhibit TPH2 activity in dorsal raphe serotonergic neurons (Fig. 3b). Nevertheless, the brief exposure to PCPA resulted in a significant decrease in fetal forebrain 5-HT levels (Fig. 3b). The data demonstrate that an exogenous source of 5-HT produced in the placenta is required to maintain normal levels of forebrain 5-HT during early stages of forebrain development (Supplementary Fig. 5).

The concept that 5-HT from maternal blood could be transferred to the fetal circulation after crossing the placenta is widely accepted[1,2] but direct or indirect transfer of the molecule has never been demonstrated. Although uptake of exogenous 5-HT by syncytiotrophoblasts in the human and mouse placenta was demonstrated *in vitro*[2,21], the concomitant high level of *Maoa* expression suggests that the placenta would prevent the vasoconstrictive effect of any free maternal blood 5-HT (a small fraction of total blood 5-HT[19]) rather than transfer it to the fetus[21]. This potentially lethal bioactivity and the quasi-absence of maternal blood 5-HT transfer to the fetus has been demonstrated previously[20,22] and also in this study. A recent study concluded that maternal 5-HT is crucial for early fetal development (before E11[1]) based on abnormal phenotypes that emerge in embryos of *Tph1*$^{-/-}$ pregnant dams. Indirect effects of the *Tph1*$^{-/-}$ maternal mutation, however, cannot be excluded as *Tph1*$^{-/-}$ mice are diabetic[23], a pathological condition that affects fetal development independently of a direct maternal 5-HT effect[24]. Although the importance of a maternal source of 5-HT before and during placentation remains an open question, our results provide the first direct evidence for maternal influences on fetal brain development through a precursor that is metabolized directly by the placenta. Given our demonstration of synthetic capability

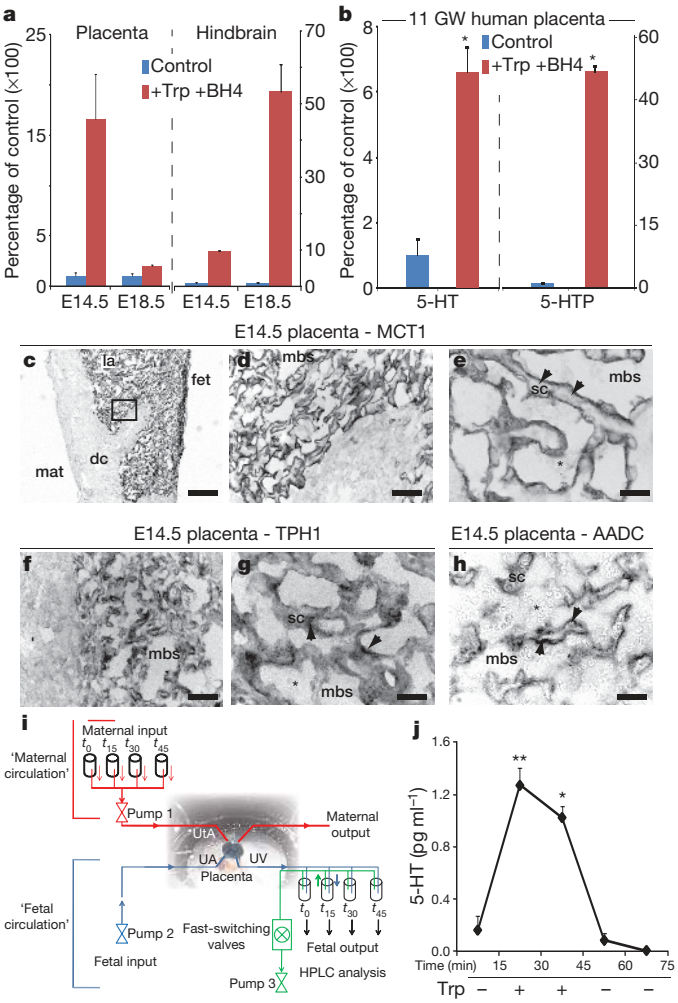

Figure 2 | Placental synthesis of 5-HT *in vitro* and *ex vivo*. **a**, Placental and hindbrain extracts were incubated with the cofactor tetrahydrobiopterin (BH4; control) or BH4 and tryptophan (+Trp+BH4) and 5-HT neosynthesis was measured after 30 min. **b**, Similar experiments conducted using human placenta tissue collected at 11 weeks of gestation (11 GW) show that human fetal villi synthesize 5-HTP and 5-HT (statistical significance versus control analysed by Student's *t*-test; *, $P < 0.005$; $n = 3$; data are presented as means ± s.e.m.). **c**, Immunostaining for the monocarboxylate transporter MCT1, a marker of syncytiotrophoblastic cells (sc) in the labyrinth (la) region on the fetal side (fet) of an E14.5 mouse placenta. On the maternal side (mat) the decidua (dc) is devoid of staining. **d**–**h**, Higher magnifications of the region boxed in **c**; MCT1 is expressed on the apical side (arrows) of syncytiotrophoblasts facing the maternal blood space (mbs) (**d**, **e**). The 5-HT synthetic enzymes TPH1 (**f**, **g**) and AADC (**h**) are expressed in overlapping patterns in the cytoplasm of syncytiotrophoblastic cells (arrows). Asterisks (**e**, **g**, **h**) indicate red blood cells of maternal origin. Scale bars: **c**, 20 mm; **d**, **f**: 500 μm; **e**, **g**, **h**: 150 μm. **i**, Schematics of the *ex vivo* dual perfusion system for the mouse placenta. UA, umbilical artery; UtA, uterine artery; UV, umbilical vein. **j**, 5-HT neosynthesis in *ex vivo* dually perfused mouse placentas at E17.5. L-tryptophan (100 μM) was injected through the uterine artery. 5-HT, neo-synthesized from maternal tryptophan and released into the umbilical vein, is evident within 15 min of precursor injection (statistical significance of 5-HT levels variation across time was analysed by one-way ANOVA; *, $P < 0.05$; **, $P < 0.01$; data from three independent experiments are presented as means ± s.e.m.).

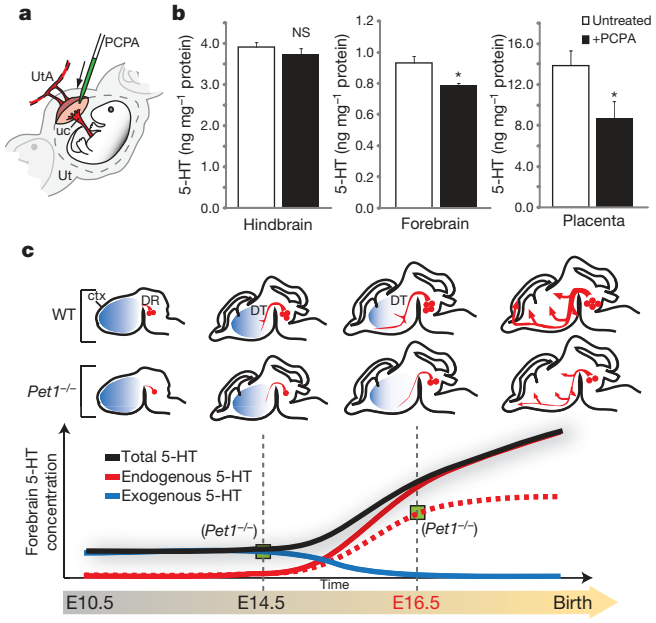

Figure 3 | HPLC measures of 5-HT concentrations in E14.5 hindbrain, forebrain and placenta of *in utero* PCPA-injected mice. **a**, Illustration of the *in vivo* injection procedure: PCPA or vehicle solution was injected into the labyrinth zone of the placenta. After 30 min, dams were killed and tissue collected and processed for HPLC. Uc, umbilical cord; Ut, uterus; UtA, uterine artery. **b**, Compared to vehicle injection (untreated, $n = 6$), placental PCPA injection (+PCPA, $n = 4$) has no significant (NS) effect on 5-HT concentration in the hindbrain, whereas it significantly lowers 5-HT concentration in the forebrain and the placenta (statistical significance versus 5-HT levels in untreated tissue was analysed by Student's *t*-test; *, $P < 0.05$; data are presented as means ± s.e.m.). **c**, Model of the progressive switch of the source of 5-HT in the fetal forebrain, from an early exogenous (placental, blue line) to a later endogenous (5-HT axons, red line) source. The green boxes represent the amount of 5-HT measured in the *Pet1*$^{-/-}$ mice, which lack most of the endogenous neuronal source. ctx, cortex; DR, dorsal raphe; DT, dorsal thalamus.

in the early human placenta, it will be clinically important to define the specific time period during human pregnancy for this placental influence on brain development. The present results also emphasize the need to examine fetal and placental tryptophan availability. Mutations in tryptophan 2,3-dioxygenase degrading enzymes (TDO1 and TDO2, which are expressed in the placenta[25]) affect neurogenesis, produce anxiety-related behaviour in mice[26] and are associated with increased risks of schizophrenia, bipolar disorder and autism[27,28]. Our results provide a mechanism through which alterations of tryptophan metabolic pathways in the placenta would affect placental 5-HT synthesis and fetal forebrain development.

Our study provides a new framework for understanding the complexity of maternal-fetal relationships that can influence brain structure and function. We focused on the 5-HT system and showed that there is a progressive switch from an early dependence on an exogenous (placental) source of 5-HT to a later endogenous brain source (Fig. 3c). The exogenous source of 5-HT is provided to the forebrain during developmental epochs that include cortical neurogenesis, migration and initial axon targeting[10]. These events can be negatively affected by disrupting 5-HT signalling: we demonstrated that a forebrain-specific disruption of 5-HT signalling *in vivo* affects axon guidance, leading to abnormal thalamocortical axon trajectories[4]. This phase in the mouse corresponds to the first and early second trimesters in the human: prenatal periods that are associated with greater risk for mental illnesses due to maternal perturbations[29,30]. Thus, translation of our findings to those corresponding periods in the human will be of significant clinical relevance.

METHODS SUMMARY

Animals and reagents. Timed-pregnant CD-1 mice were purchased from the Charles River Laboratory. Plug date was considered E0.5 and the age of individual embryos was confirmed by measuring the crown–rump length and checking for developmental landmarks such as digits and eye formation. *Pet1* (ref. 3) knockout (−/−), heterozygotes (+/−) and wild-type (+/+) littermate embryos were generated by crossing $Pet1^{+/-}$ males and females. $Sert^{+/-}$ embryos were obtained by crossing $Sert^{-/-}$ females[19] with wild-type C57BL/6 males. The *Pet1* and *Sert* knockout lines have been backcrossed on the C57Bl/6J background for >10 generations. The *Maoa* spontaneous knockout mouse (s*Maoa* KO) was described earlier[16]. All procedures using mice were approved by the Institutional Animal Care and Use Committee at University of Southern California and conformed to NIH guidelines. Unless otherwise noted, all reagents and antibodies were purchased from Sigma. Protocols for HPLC, tissue staining, *in vitro* 5-HT synthesis assays, MAOA enzymatic activity assays, *in vivo* synthesis inhibition assays and *ex vivo* placental perfusions are described in Methods.

Human placenta samples. Normal human placental villi samples were obtained from women undergoing elective pregnancy terminations at the Reproductive Options Clinic at the Los Angeles County (LAC) and University of Southern California (USC) Medical Center. For the purposes of this study, the tissue was carefully dissected by the pathologist and immediately flash-frozen in liquid nitrogen. No patient characteristics other than gestational age were recorded. The University of Southern California Health Science Campus Institutional Review Board approved this study.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 24 August 2010; accepted 24 February 2011.

1. Cote, F. *et al.* Maternal serotonin is crucial for murine embryonic development. *Proc. Natl Acad. Sci. USA* **104,** 329–334 (2007).
2. Yavarone, M. S., Shuey, D. L., Sadler, T. W. & Lauder, J. M. Serotonin uptake in the ectoplacental cone and placenta of the mouse. *Placenta* **14,** 149–161 (1993).
3. Hendricks, T. J. *et al.* Pet-1 ETS gene plays a critical role in 5-HT neuron development and is required for normal anxiety-like and aggressive behavior. *Neuron* **37,** 233–247 (2003).
4. Bonnin, A., Torii, M., Wang, L., Rakic, P. & Levitt, P. Serotonin modulates the response of embryonic thalamocortical axons to netrin-1. *Nature Neurosci.* **10,** 588–597 (2007).
5. Ansorge, M. S., Zhou, M., Lira, A., Hen, R. & Gingrich, J. A. Early-life blockade of the 5-HT transporter alters emotional behavior in adult mice. *Science* **306,** 879–881 (2004).
6. Gross, C. *et al.* Serotonin1A receptor acts during development to establish normal anxiety-like behaviour in the adult. *Nature* **416,** 396–400 (2002).
7. Taylor, S. E. *et al.* Early family environment, current adversity, the serotonin transporter promoter polymorphism, and depressive symptomatology. *Biol. Psychiatry* **60,** 671–676 (2006).
8. Bonnin, A., Peng, W., Hewlett, W. & Levitt, P. Expression mapping of 5–HT1 serotonin receptor subtypes during fetal and early postnatal mouse forebrain development. *Neuroscience* **141,** 781–794 (2006).
9. Anderson, G. M. *et al.* Serotonin transporter promoter variants in autism: functional effects and relationship to platelet hyperserotonemia. *Mol. Psychiatry* **7,** 831–836 (2002).
10. Gaspar, P., Cases, O. & Maroteaux, L. The developmental role of serotonin: news from mouse molecular genetics. *Nature Rev. Neurosci.* **4,** 1002–1012 (2003).
11. Buznikov, G. A., Lambert, H. W. & Lauder, J. M. Serotonin and serotonin-like substances as regulators of early embryogenesis and morphogenesis. *Cell Tissue Res.* **305,** 177–186 (2001).
12. Aitken, A. R. & Tork, I. Early development of serotonin-containing neurons and pathways as seen in wholemount preparations of the fetal rat brain. *J. Comp. Neurol.* **274,** 32–47 (1988).
13. Lidov, H. G. & Molliver, M. E. Immunohistochemical study of the development of serotonergic neurons in the rat CNS. *Brain Res. Bull.* **9,** 559–604 (1982).
14. Lebrand, C. *et al.* Transient uptake and storage of serotonin in developing thalamic neurons. *Neuron* **17,** 823–835 (1996).
15. Lebrand, C. *et al.* Transient developmental expression of monoamine transporters in the rodent forebrain. *J. Comp. Neurol.* **401,** 506–524 (1998).
16. Scott, A. L., Bortolato, M., Chen, K. & Shih, J. C. Novel monoamine oxidase A knock out mice with human-like spontaneous mutation. *Neuroreport* **19,** 739–743 (2008).
17. Lynn-Bullock, C. P., Welshhans, K., Pallas, S. L. & Katz, P. S. The effect of oral 5-HTP administration on 5-HTP and 5-HT immunoreactivity in monoaminergic brain regions of rats. *J. Chem. Neuroanat.* **27,** 129–138 (2004).
18. Branchek, T. A. & Gershon, M. D. Time course of expression of neuropeptide Y, calcitonin gene-related peptide, and NADPH diaphorase activity in neurons of the developing murine bowel and the appearance of 5-hydroxytryptamine in mucosal enterochromaffin cells. *J. Comp. Neurol.* **285,** 262–273 (1989).
19. Chen, J. J. *et al.* Maintenance of serotonin in the intestinal mucosa and ganglia of mice that lack the high-affinity serotonin transporter: Abnormal intestinal motility and the expression of cation transporters. *J. Neurosci.* **21,** 6348–6361 (2001).
20. Howd, R. A., Nelson, M. F. & Lytle, L. D. L-tryptophan and rat fetal brain serotonin. *Life Sci.* **17,** 803–811 (1975).
21. Balkovetz, D. F., Tiruppathi, C., Leibach, F. H., Mahesh, V. B. & Ganapathy, V. Evidence for an imipramine-sensitive serotonin transporter in human placental brush-border membranes. *J. Biol. Chem.* **264,** 2195–2198 (1989).
22. Robson, J. M. & Senior, J. B. The 5-hydroxytryptamine content of the placenta and foetus during pregnancy in mice. *Br. J. Pharmacol. Chemother.* **22,** 380–391 (1964).
23. Kim, H. *et al.* Serotonin regulates pancreatic beta cell mass during pregnancy. *Nature Med.* **16,** 804–808 (2010).
24. Jawerbaum, A. & White, V. Animal models in diabetes and pregnancy. *Endocr. Rev.* **31,** 680–701 (2010).
25. Suzuki, S. *et al.* Expression of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase in early concepti. *Biochem. J.* **355,** 425–429 (2001).
26. Kanai, M. *et al.* Tryptophan 2,3-dioxygenase is a key modulator of physiological neurogenesis and anxiety-related behavior in mice. *Mol. Brain* **2,** 8 doi:10.1186/1756-6606-2-8 (2009).
27. Miller, C. L. *et al.* Two complex genotypes relevant to the kynurenine pathway and melanotropin function show association with schizophrenia and bipolar disorder. *Schizophr. Res.* **113,** 259–267 (2009).
28. Nabi, R., Serajee, F. J., Chugani, D. C., Zhong, H. & Huq, A. H. Association of tryptophan 2,3 dioxygenase gene polymorphism with autism. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* **125B,** 63–68 (2004).
29. Brown, A. S. & Derkits, E. J. Prenatal infection and schizophrenia: a review of epidemiologic and translational studies. *Am. J. Psychiatry* **167,** 261–280 (2010).
30. Oberlander, T. F., Gingrich, J. A. & Ansorge, M. S. Sustained neurobehavioral effects of exposure to SSRI antidepressants during development: molecular to clinical evidence. *Clin. Pharmacol. Ther.* **86,** 672–677 (2009).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank H.H. Wu and K. Eagleson for discussions and comments on the manuscript. We thank R. Johnson (Vanderbilt HPLC core facility), L. Zhang for technical help and E. Meng for discussions. This work was supported by the NICHD (grant 5R21HD065287 to A.B.), NARSAD (A.B.) and the NIMH (grant R01MH39085 to J.C.S. and 1P50MH078280A1 to R.D.B. and P.L.).

Author Contributions A.B. conducted the experiments with assistance from N.G. in placenta studies, K.C. and J.C.S. in providing mutant mouse strains and in MAOA enzymatic assays. R.D.B. and E.S.D. provided mutant mouse strains and M.L.W. and J.K. provided human tissue. A.B. and P.L. conceived this study, interpreted the data and wrote the manuscript. All authors commented on the paper.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to A.B. (bonnin@usc.edu) or P.L. (plevitt@usc.edu).

METHODS

Animals and reagents. Timed-pregnant CD-1 mice were purchased from the Charles River Laboratory. Plug date was considered E0.5 and the age of individual embryos was confirmed by measuring the crown–rump length and checking for developmental landmarks such as digits and eye formation. *Pet1* (ref. 3) knockout (−/−), heterozygotes (+/−) and wild-type (+/+) littermate embryos were generated by crossing $Pet1^{+/-}$ males and females. $Sert^{+/-}$ embryos were obtained by crossing $Sert^{-/-}$ females[19] with wild-type C57BL/6 males. The *Pet1* and *Sert* knockout lines have been backcrossed on the C57Bl/6J background for >10 generations. The *Maoa* spontaneous knockout mouse (s*Maoa* KO) was described earlier[16]. All procedures using mice were approved by the Institutional Animal Care and Use Committee at University of Southern California and conformed to NIH guidelines. Unless otherwise noted, all reagents and antibodies were purchased from Sigma.

Human placenta samples. Normal human placental villi samples were obtained from women undergoing elective pregnancy terminations at the Reproductive Options Clinic at the Los Angeles County (LAC) and University of Southern California (USC) Medical Center. For the purposes of this study, the tissue was carefully dissected by the pathologist and immediately flash-frozen in liquid nitrogen. No patient characteristics other than gestational age were recorded. The University of Southern California Health Science Campus Institutional Review Board approved this study.

HPLC measures. Hindbrains and forebrains were homogenized, using a tissue dismembrator, in 100–750 µl of 0.1 M trichloroacetic acid TCA, which contains 10^{-2} M sodium acetate, 10^{-4} M EDTA, 5 ngml^{-1} isoproterenol (as internal standard) and 10.5% methanol (pH 3.8). Samples were spun at 10,000g for 20 min. The supernatant was removed and stored at −80 °C. The pellet was used for protein analysis; total protein concentrations were determined using the BCA Protein Assay Kit (Pierce Chemical Company). The supernatant was then thawed and spun for 20 min. The concentration of biogenic amines in each sample was determined by a specific HPLC assay using an Antec Decade II (oxidation, 0.5 V) electrochemical detector operated at 33 °C. 20 µl samples of supernatant were injected using a Water 717+ autosampler onto a PhenomenexNucleosil (5 u, 100 A) C18 HPLC column (150 × 4.60 mm). Biogenic amines were eluted with a mobile phase consisting of 89.5% 100 mM TCA, 10 mM sodium acetate, 0.1 mM EDTA and 10.5% methanol (pH 3.8). Solvent was delivered at 0.6 mlmin^{-1} using a Waters 515 HPLC pump. Using this HPLC solvent, the biogenic amines eluted in the following order: noradrenaline, adrenaline, DOPAC, dopamine, 5-HIAA, HVA, 5-HT and 3-MT. HPLC control and data acquisition were managed by Millennium 32 software.

Statistical analysis. Measures in $Pet1^{-/-}$ and wild-type littermate embryos ($n \geq 6$ per genotype) are presented as mean ± s.e.m. Statistical comparison of measures between genotypes across development was performed using a one-way ANOVA (Fig. 1). Differences were considered statistically significant at $P < 0.05$. Single statistical comparison between two groups was performed using a two-tailed Student's *t*-test (for example, comparison of measures in $Pet1^{-/-}$ and wild-type littermates or s$Maoa^{-/-}$ and wild-type littermates at a single age or *in utero* PCPA- versus vehicle-injected mice; see Fig. 3 and Supplementary Tables 1 and 2).

***In vitro* 5-HT synthesis assays.** Pregnant CD-1 mice were anaesthetized by an intraperitoneal injection of 0.2 ml pentobarbital and perfused through the left ventricle with 100 ml of phosphate buffered saline (PBS). The uterus was immediately dissected and the resulting embryos were placed on ice in PBS. The placenta, forebrain and hindbrain were removed and kept on ice in 1.5 ml eppendorf tubes. The collected tissue was homogenized in 3 volumes of 0.05 M Tris buffer, pH 7.5, containing 1 mM dithiothreitol and 1 mM EGTA. Homogenates were centrifuged at 29,000g for 15 min at 4 °C and the supernatants were placed on ice; tryptophan hydroxylase assays were performed as previously described[31]. Briefly, 70 µg of protein from the supernatant was added to tubes containing 100 µl of: 0.05 M Tris buffer, pH 7.5, 1 mM EGTA, 15 µg catalase, 250 µM L-tryptophan, and 50 µM tetrahydrobiopterin (BH4; a cofactor required for TPH1 and TPH2 activity[32]). Reactions were performed for 10 and 30 min at 37 °C and stopped by flash freezing in liquid nitrogen. L-tryptophan metabolism was determined by measuring 5-hydroxytryptophan (5-HTP), 5-hydroxytyptamine (5-HT), and 5-hydroxyindoleacetic acid (5-HIAA) using HPLC with electrochemical detection (see above). Protein concentrations were determined using a DC protein assay (Bio-Rad).

***In vitro* MAO-A activity measures.** MAO-A activity was determined by incubating forebrain and hindbrain homogenates with [^{14}C]-5-HT (1 mM) at 37 °C for 20 min. At the end of the incubation, the products were extracted and the radioactivity determined as described previously[33].

Immunohistochemistry. Brain sections were processed for immunohistochemistry using the following primary antibodies: anti-5-HT (rabbit, 1:1,000; goat, 1:1,000, Sigma), anti-NetrinG1a (goat, 1:250, RnD), anti-SERT (rabbit, 1:200, Sigma), anti-5-HTP (rabbit, 1:1,500; ImmunoStar) and anti-AADC (rabbit, 1:500, Abcam). The secondary antibodies (Jackson ImmunoResearch) were: Cy2- or Cy3-conjugated donkey anti-rabbit IgG (1:800), rhodamin-X conjugated donkey anti-goat (1:200) and biotin-conjugated anti-rabbit (1:1,000). 20 µm brain sections were permeabilized, and pre-incubated with blocking solution (0.1% Triton X-100, 2% fetal bovine serum) in PBS for 2 h and then incubated overnight with a primary antibody diluted in the same blocking solution at 4 °C. Sections were washed with PBS + 0.1% Triton X-100 and incubated with appropriate secondary antibodies diluted in same blocking solutions for a minimum of 2 h at room temperature (22-25 °C). Sections were then washed with PBS + 0.1% Triton X-100 and mounted using a fluorescence anti-fade medium (Prolong Gold, Vector Laboratories). For non-fluorescent immunohistochemistry (for example, AADC staining), similar procedures were followed, except that the sections were incubated in methanol with 3% H_2O_2 for 15 min to quench endogenous peroxidase activity. The reaction product was visualized using a Vector ABC Elite Kit (Vector Laboratories). Images were acquired on a Zeiss AxoplanII microscope coupled to an AxioCamHRc camera using Axiovision 4.1 software (Zeiss). For placental immunochemistry, 5 µm frozen sections were thawed for 15 min at room temperature then washed in a mixture of 1× PBS containing 0.1% Triton X-100 (PBS-T) for 15 min at room temperature to allow for membrane permeabilization. Endogenous HRP was quenched by incubating the slides in a solution of methanol containing 3% hydrogen peroxide for 15 min at room temperature. The slides were then incubated for 2 h in a solution of PBS-T containing 2% Fetal Bovine Serum (Blocking Solution). The slides were then incubated overnight with anti-MCT1 (MCT1 is a marker of syncytiotrophoblasts[34]; chicken; 0.5 µgml^{-1}) or anti-AADC or anti-TPH1 (rabbit; 1:500) overnight at room temperature. Slides were then washed 3 × 10 min in PBS-T and incubated with appropriate secondary antibodies (biotin-conjugated anti-chicken; 1:1,000 or biotin-conjugated anti-rabbit; 1:1,000; Jackson ImmunoResearch) for 2 h at room temperature. After 6 × 5 min washes, slides were processed for HRP visualization as described above.

***In utero* inhibition of placental TPH1.** Pregnant CD-1 mice were anaesthetized by an intraperitoneal injection of 0.5–0.7 ml of 10% Nembutal and uterine horns exposed as previously described[4]. Glass micropipettes were filled with a solution of 10 mM PCPA (4-Chloro-DL-phenylalanine; an inhibitor of TPH1 enzymatic activity[35]) in PBS and 0.1% fast green dye (FCF; Sigma). FCF was used to monitor visually the diffusion of solution into the fetal villi of the placenta. 2–5 µl were injected through the uterine wall into the labyrinth region in half of the placentas ($n = 4$–6) for each pregnant mouse ($n = 3$); remaining placentas were injected with vehicle only. After 30 min, the dam was killed and placentas and corresponding forebrains and hindbrains collected and quickly frozen in liquid N_2. Samples were then processed for HPLC analysis as described above.

***Ex vivo* dual perfusion of the mouse placenta.** A single placenta with uterine and umbilical arteries and veins attached was harvested from a pregnant CD-1 mouse at E17.5 in warm PBS. The placenta was rapidly transferred to a thermostated tissue culture chamber (60 mm diameter, Warner Instruments) to which warm (37 °C) and oxygenated (95% O_2, 5% CO_2) PBS solution was constantly provided. Using a dissection microscope, micro-catheters were carefully attached to the uterine artery on the maternal side and umbilical artery and vein on the fetal side of the placenta. The uterine artery at E17.5 (internal diameter is approximately 150 µm) was cannulated using a 150 µm outside diameter polyethylene micro-catheter. Micro-catheters were kept solidly attached to the arteries or veins using micro-clamps (F.S.T.) and suture. The other end of the uterine artery micro-catheter was connected to an 8-channel micro-manifold (Automate Scientific); each channel's polyethylene tubing was then connected to an individual fast-switching valve (1.5 ms response time; Automate Scientific); the artificial maternal solution (warm oxygenated PBS) was delivered to each individual channel through a pressurized cylinder connected to each valve. An electronic valve controller enabled timed sequential switching of maternal solutions. The input pressure was calibrated to allow a maximal flow rate of 10–20 µlmin^{-1} through the uterine artery micro-catheter. On the fetal side, the umbilical artery and vein were similarly cannulated; for E17.5 placentas polyethylene tubing with 100 µm internal diameter was used. The other end of the umbilical artery micro-catheter was directly connected to a 5 ml syringe containing the artificial fetal solution (PBS with 0.01% fast green dye FCF, Sigma) and mounted on a precision, computer-controlled syringe pump (Braintree Scientific); the infusion flow rate was set at 6 µlmin^{-1}. For fetal eluate collection, the umbilical vein was cannulated to 100 µm internal diameter polyethylene tubing; this tubing was then connected to a specially modified SmartSquirt system (Automate Scientific) which consisted of a micro-manifold connected to eight independent 3-ml reservoirs in which a controlled negative pressure (−15 PSI) was individually applied. The integrated fast-switching valves system allowed the application of negative pressure to each reservoir

independently and sequentially, thus allowing individual fraction collection over time. The addition of fast green dye to the fetal perfusate allowed monitoring for any leak through and around the placenta during the perfusion.

31. Johansen, P. A., Jennings, I., Cotton, R. G. & Kuhn, D. M. Tryptophan hydroxylase is phosphorylated by protein kinase A. *J. Neurochem.* **65,** 882–888 (1995).
32. Kuhn, D. M., Ruskin, B. & Lovenberg, W. Tryptophan hydroxylase. The role of oxygen, iron, and sulfhydryl groups as determinants of stability and catalytic activity. *J. Biol. Chem.* **255,** 4137–4143 (1980).
33. Chen, K. *et al.* Forebrain-specific expression of monoamine oxidase A reduces neurotransmitter levels, restores the brain structure, and rescues aggressive behavior in monoamine oxidase A-deficient mice. *J. Biol. Chem.* **282,** 115–123 (2007).
34. Nagai, A., Takebe, K., Nio-Kobayashi, J., Takahashi-Iwanaga, H. & Iwanaga, T. Cellular expression of the monocarboxylate transporter (MCT) family in the placenta of mice. *Placenta* **31,** 126–133 (2010).
35. Koe, B. K. & Weissman, A. p-Chlorophenylalanine: a specific depletor of brain serotonin. *J. Pharmacol. Exp. Ther.* **154,** 499–516 (1966).