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Monoaminergic regulation of Sonic hedgehog signaling cascade expression in the adult rat hippocampus

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ABSTRACT

Monoamines are implicated in the modulation of adult hippocampal neurogenesis in depression models and following chronic antidepressant treatment. Given the key role of Sonic hedgehog (Shh) in adult neurogenesis, we examined whether monoaminergic perturbations regulate the expression of Shh or its co-receptors Smoothened (Smo) and Patched (Ptc). Combined depletion of both serotonin and norepinephrine with para-chlorophenylalanine (PCPA) resulted in a significant decrease in Smo and Ptc mRNA within the dentate gyrus subfield of the hippocampus. However, selective depletion of serotonin, using the serotonergic neurotoxin 5,7-dihyrdroxytryptamine (5,7-DHT), or norepinephrine, using the noradrenergic neurotoxin DSP-4, did not alter expression of Shh and its co-receptors, Smo and Ptc. Acute treatment with the monoamine releasing agent, para-chloroamphetamine (PCA) significantly upregulated Smo mRNA within the dentate gyrus. However, acute or chronic treatment with pharmacological antidepressants that modulate monoaminergic neurotransmission did not regulate Shh cascade expression. These results indicate that robust changes in monoamine levels can regulate the expression of the Shh signaling cascade in the adult rodent brain.

Animal models of depression exhibit decreased adult hippocampal neurogenesis [19,27], whereas sustained antidepressant administration increases hippocampal neurogenesis [18] and is thought to contribute to the behavioral effects of antidepressants [23]. The monoaminergic hypothesis suggests that decreased monoamines underlie the pathogenesis of depression, and increased monoamines contribute to antidepressant action [6]. Recently monoaminergic neurotransmission has been implicated in the effects of depression models and antidepressants on adult hippocampal neurogenesis [23,26]. A decline in hippocampal neurogenesis is observed following serotonin (5-HT) and/or norepinephrine (NE) depletion, while increased hippocampal neurogenesis is seen following elevation of monoamine levels [4,9,12,18]. However, the pathways that underlie the effects of monoamines on adult hippocampal neurogenesis are not understood.

Sonic hedgehog (Shh) is a powerful regulator of adult hippocampal neurogenesis and is essential for the maintenance of the adult stem cell niches [14,17,21]. In the adult brain, Shh is expressed in the neocortex and the horizontal and vertical limbs of the diagonal band from where it is thought to be anterogradely transported to the hippocampus [24,25]. Within the hippocampus, expression of the Shh co-receptors Patched (Ptc) and Smoothened (Smo) is seen in the dentate gyrus subfield including the neurogenic niche of subgranular zone (SGZ) [14,24]. Smo protein has also been recently shown to localize predominantly to mossy fiber nerve terminals in the hippocampus [20]. Recent evidence indicates that the neurogenic effects of electroconvulsive seizure (ECS) treatment, one of the most robust forms of antidepressant therapy, requires Shh signaling and ECS also regulates the hippocampal expression of Smo and Ptc [1]. We hypothesized that monoaminergic neurotransmission may influence the expression of the Shh cascade. The aim of the present study was to address whether robust perturbations in the levels of 5-HT and NE or treatment with pharmacological antidepressants regulate the expression of key components of the Shh signaling cascade.

Male Sprague–Dawley rats (200–250 g; TIFR animal breeding colony) were group-housed and maintained on a controlled 12 h light/dark cycle with access to food and water *ad libitum*. All animal procedures were carried out in accordance with the NIH *Guide for the Care and Use of Laboratory Animals*, and were approved by the TIFR Institutional Animal Ethics Committee.

To induce a combined depletion of norepinephrine (NE) and serotonin (5-HT), animals were treated with p-chlorophenylalanine (PCPA, 300 mg/kg—2 days, 100 mg/kg—2 days; Sigma, USA) as

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previously described [4,9]. To induce a selective depletion of NE, animals were treated with N-Ethyl-N-(2-chloroethyl)-2-bromobenzylamine hydrochloride (DSP-4, 10 mg/kg, Sigma) using a previously described protocol [12]. To induce a selective depletion of 5-HT, animals received an intracerebroventricular (i.c.v.) infusion (AP-0.8 mm, ML-1.4 mm and DV-4.0 mm from Bregma [22]) of 5,7-dihydroxytryptamine (5,7-DHT, 200 μg/animal, 20 μg/μl, Sigma) as reported previously [9]. The choice of dose, treatment paradigm and time-point for sacrifice for the above studies was based on prior literature that indicated a robust depletion of the specific monoamines [4,9,12]. To induce a robust monoamine release, animals received p-Chloroamphetamine (PCA, 10 mg/kg, Sigma) or vehicle (0.9% saline) through intraperitoneal (i.p.) injection and were sacrificed 2 h later. For the acute antidepressant experiment, animals received an i.p. injection of fluoxetine (FLX; 5 mg/kg), tranylcypromine (TCP; 7.5 mg/kg), desipramine (DMI; 15 mg/kg) or vehicle (0.9% saline) and were sacrificed 2 h later. For the chronic antidepressant experiment, animals received i.p. injections of FLX (5 mg/kg), TCP (7.5 mg/kg for 7 days and 10 mg/kg for 14 days), DMI (15 mg/kg) or vehicle (0.9 % saline) once daily for 21 days and were sacrificed 2 h after the last treatment. The choice of doses for antidepressant treatments was based on prior literature that indicated an effect on adult hippocampal progenitor proliferation at these doses [18]. All antidepressant drugs were obtained from Sigma. After decapitation, brains were frozen prior to cryostat sectioning and *in situ* hybridization analysis.

In situ hybridization was carried out as previously described [1] on 14 μm thick coronal sections. Slides were fixed, acetylated and dehydrated. Rat Shh, Smo and Ptc cRNA probes were transcribed using ^{35}S-labeled UTP (Amersham) from plasmids provided by Dr. Martial Ruat (CNRS, France). Slides were incubated with radiolabeled riboprobes (1×10^6 cpm/150 μl per slide) in hybridization buffer overnight and then subjected to stringent post-hybridization washes. Slides were exposed to Hyperfilm β-max (Amersham) for 2–3 weeks. Shh, Ptc or Smo sense riboprobes did not yield significant hybridization (data not shown) confirming the specificity of the signal observed with the antisense riboprobes. Shh, Ptc and Smo mRNA levels were quantitated with the Macintosh-based Scion Image software (Scion, USA) using ^{14}C standards (Amersham, USA) for calibration and to correct for non-linearity. For Shh mRNA, expression in the horizontal and vertical limbs of the diagonal band (VDB) and in the neocortex was quantitated. For Ptc and Smo mRNA, the expression in the dentate gyrus was determined. The optical density was determined by outlining an equivalent area in each section, and the means were determined from measurements taken on both sides of 4 individual sections/animal.

Quantitative real-time PCR (qPCR) was also performed for acute PCA treatment. Animals ($n = 5$/group) were treated with PCA or vehicle and sacrificed as described earlier. Hippocampi were subdissected in cold saline and frozen in liquid nitrogen. Total RNA was isolated from hippocampal tissue using Tri Reagent (Sigma), according to the manufacturer's protocol. RNA was quantified using Nanodrop (Eppendorf, Germany) and RNA quality determined by resolving on 1% formaldehyde agarose gel. cDNA was synthesized from 200 ng of total RNA per sample using Quantitect reverse transcription kit (Qiagen, USA), as per the manufacturer's protocol. cDNA was amplified and visualized using a SYBR Green kit (Applied Biosystems, USA) in a Realplex mastercycler (Eppendorf). Hypoxanthine phopshoribosyl transferase (HPRT) was used as the endogenous control. To compare HPRT and target gene, relative quantification was performed using comparative C_T method [2]. Briefly, this comparative C_T method involved averaging duplicate samples of each target and endogenous control in both control and treatment samples [i.e. ΔC_T = absolute C_T value − endogenous control C_T value and $\Delta\Delta C_T = \Delta C_T$ treatment − ΔC_T control]. The fold change was calculated according to the formula $2^{(-\Delta\Delta C_T)}$. The primer sequences used were Ptc F—CCATTTCTTGCCCTTGGTGTTGGT, R—AATGCAGCCATGAAGAAGGCAGTG; Smo F—AATTGGCCTGGTGCTTATTGTGGG, R—AGGGTGGTTGCTCTTGATGGAGAA; HPRT F—GCAGACTTTGCTTTCCTTGG, R—GTCTGGCCTGTATCCAACACT.

Experiments were analyzed for differences using the unpaired Student's *t*-test (Prism, Graphpad, USA) with significance determined at p values < 0.05.

Combined, but not selective, depletion of norepinephrine and serotonin levels downregulates Smo and Ptc mRNA levels in the dentate gyrus.

The expression of Smo, the signaling component of the Shh receptor complex, was found to be significantly decreased (~30%) in the hippocampal dentate gyrus subfield following depletion of both NE and 5-HT by PCPA treatment. In addition, there was a small but significant decrease (~20%) in the level of Ptc mRNA in the DG of PCPA treated animals as compared to the vehicle group (Fig. 1). In striking contrast, selective noradrenergic (DSP-4) and serotonergic (5,7-DHT) neurotoxin treatments did not alter Smo or Ptc mRNA expression in the hippocampus (Fig. 2). The mRNA expression of the ligand Shh in the VDB (Figs. 1 and 2) and the neocortex (PCPA treatment: Veh = 100 ± 3.27, PCPA = 98.83 ± 2.09; DSP-4 treatment: Veh = 100 ± 11.47, DSP-4 = 99.75 ± 7.22; 5,7-DHT treatment: Veh = 100 ± 5.82, 5,7-DHT = 113.09 ± 4.29; results are expressed as percent of Vehicle and are the mean ± S.E.M.) was not influenced by PCPA, DSP-4 or 5,7-DHT treatments. As previously reported [24], Shh mRNA expression was high in the VDB and was expressed at relatively lower levels in the neocortex (layer V).

Treatment with the monoamine releasing agent, PCA, increases Smo mRNA in the dentate gyrus, however pharmacological antidepressants do not regulate the Shh signaling cascade.

Acute PCA treatment resulted in a robust and significant upregulation of Smo mRNA (~65%) within the DG subfield of the hippocampus (Fig. 1). PCA treatment did not influence either the expression of Shh in the basal forebrain (Fig. 1) or neocortex (PCA Treatment, Shh mRNA levels: Veh = 100 ± 8.26, PCA = 113.35 ± 11.25, results are expressed as percent of Vehicle and are the mean ± S.E.M.) or the co-receptor Ptc in the hippocampal subfields (Fig. 1). The *in situ* regulation of Smo and Ptc mRNA were further validated by qPCR for acute PCA treatment. Acute PCA resulted in a 70% increase in Smo mRNA in the hippocampus as compared to vehicle treated controls (ΔC_T acute PCA = 0.915 ± 0.1762; ΔC_T Vehicle = 1.68 ± 0.2463; $\Delta\Delta C_T = -0.765$; results are expressed as ΔC_T and are the mean ± S.E.M.; $p < 0.05$, Student's *t*-test). Hippocampal Ptc mRNA levels were found to be unaltered following acute PCA treatment (ΔC_T acute PCA = 3.041 ± 0.3414; ΔC_T Vehicle = 3.577 ± 0.2124; $\Delta\Delta C_T = -0.536$; results are expressed as ΔC_T and are the mean ± S.E.M.; $p > 0.05$, Student's *t*-test). Further, Shh mRNA levels in the VDB and the neocortex, and Smo and Ptc mRNA levels in the DG were not altered by either acute or chronic treatment with the pharmacological antidepressants DMI, FLX or TCP (Table 1).

The present study indicates that robust changes in levels of both 5-HT and NE modulate the expression of the Shh co-receptors, Smo and Ptc, in the hippocampal dentate gyrus subfield. While depletion of both 5-HT and NE (PCPA) decreased Smo and Ptc hippocampal mRNA levels, selective depletion of either 5-HT (5,7-DHT) or NE (DSP-4) did not alter their expression. Further, acute treatment with the monoamine releasing agent PCA significantly increased Smo mRNA levels. In contrast, acute or chronic antidepressant treatment with the serotonin selective reuptake inhibitor, fluoxetine, the norepinephrine selective reuptake inhibitor, desipramine, and the monoamine oxidase inhibitor, tranylcypromine did not regulate the expression of the Shh cascade. Our study provides novel evidence