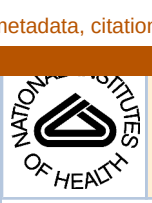

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Ontogeny of Serotonin and Serotonin$_{2A}$ Receptors in Rat Auditory Cortex

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Abstract

Maturation of the mammalian cerebral cortex is, in part, dependent upon multiple coordinated afferent neurotransmitter systems and receptor-mediated cellular linkages during early postnatal development. Given that serotonin (5-HT) is one such system, the present study was designed to specifically evaluate 5-HT tissue content as well as 5-HT$_{2A}$ receptor protein levels within the developing auditory cortex (AC). Using high performance liquid chromatography (HPLC), 5-HT and the metabolite, 5-hydroxyindoleacetic acid (5-HIAA), was measured in isolated AC, which demonstrated a developmental dynamic, reaching young adult levels early during the second week of postnatal development. Radioligand binding of 5-HT$_{2A}$ receptors with the 5-HT$_{2A/2C}$ receptor agonist, ^{125}I-DOI ((+/−)-1-(2,5- dimethoxy-4-iodophenyl)-2-aminopropane HCl; in the presence of SB206553, a selective 5-HT$_{2C}$ receptor antagonist, also demonstrated a developmental trend, whereby receptor protein levels reached young adult levels at the end of the first postnatal week (P8), significantly increased at P10 and at P17, and decreased back to levels not significantly different from P8 thereafter. Immunocytochemical labeling of 5-HT$_{2A}$ receptors and confocal microscopy revealed that 5-HT$_{2A}$ receptors are largely localized on layer II/III pyramidal cell bodies and apical dendrites within AC. When considered together, the results of the present study suggest that 5-HT, likely through 5-HT$_{2A}$ receptors, may play an important role in early postnatal AC development.

Keywords

Auditory Cortex; High Performance Liquid Chromatography; Immunocytochemistry; Serotonin; Serotonin 2A Receptors; Autoradiography

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Introduction

The mature organization of cerebral cortical circuits depends upon the coordinated formation of synapses from multiple afferent neurotransmitter systems during development. For example, auditory cortical neurons receive input from many afferent systems that ultimately influence the formation of cortical circuits beginning in gestation, including serotonin (5-hydroxytryptamine; 5-HT) innervation from the brainstem raphe nuclei (Lidov and Molliver 1982; Wallace et al. 1982a; Wallace and Lauder 1983b). Serotonin may play a particularly important role in auditory cortex (AC), as loudness growth functions have been reported to depend on 5-HT levels (Hegerl and Juckel 1993), central auditory plasticity produced by fear conditioning relies on 5-HT (Ji and Suga 2007), and significant changes in acoustically evoked AC activity have been reported with altered brain 5-HT levels in humans and rats (Kahkonen et al. 2002a; Kahkonen et al. 2002b; Manjarrez et al. 2005). Serotonin may also play a role in development of the auditory brainstem, as suggested by transient expression of the 5-HT transporter (5-HTT) during postnatal development of auditory nuclei, including the ventral cochlear nucleus (VCN) and principal nuclei of the superior olivary complex (Thompson and Lauder, 2005), as well as the presence of 5-HT in neurons of the lateral superior olivary nucleus during a narrow window (days 1–8) of postnatal development (Thompson, 2006).

The serotonergic system is thought to influence many processes during brain development, including neurogenesis, programmed cell death, cell migration, dendritic and axonal development, synaptogenesis, and synaptic plasticity (reviewed by Lauder, 1990). Serotonergic afferents arrive early during cortical plate development (Dori et al. 1996; Lidov and Molliver 1982; Wallace and Lauder 1983b; Whitaker-Azmitia 2001), and are densely distributed in the primary visual and somatosensory cortices of rats, mice and hamsters during the first two postnatal weeks (Bennett-Clarke et al. 1993a; Bennett-Clarke et al. 1996b). Moreover, 5-HT immunoreactive axons form a transient pattern corresponding to thalamocortical axons in lamina IV of the rat primary somatosensory cortex during early postnatal development (D'Amato et al. 1987; Rhoades et al. 1990; Stojic et al. 1998) due to 5-HT uptake and vesicular storage by thalamocortical axons (Lebrand et al. 1996).

Serotonin exerts its biological effects by activating more than fourteen 5-HT receptor subtypes (Hoyer et al. 2002), many of which are expressed in the developing cerebral cortex. Serotonin$_{1A}$, 5-HT$_{2B}$, and 5-HT$_3$ receptors are localized to the ventricular zone (Johnson and Heinemann 1995), whereas 5-HT$_{2A}$ and 5-HT$_3$ receptors are expressed by post-mitotic neurons of the cerebral cortex (Johnson and Heinemann 1995; Vitalis and Parnavelas 2003). To date, the specific regulation of AC activity by 5-HT receptors is largely unexplored, although 5-HT has been suggested to be involved in the development of other sensory areas, such as the barrel fields of the somatosensory cortex (reviewed by Luo et al. 2003). Serotonin receptors have also been proposed to play a role in regulating critical periods in visual cortex (Edagawa et al., 2001; Gu and Singer, 1995). Recent electrophysiological data indicate that 5-HT receptors regulate excitability of cortical pyramidal neurons in brain slices from postnatal days 6–19 (Beique et al. 2004). This study demonstrated robust depolarization of layer V frontal cortical pyramidal neurons, which shifted to hyperpolarization during the third postnatal week, due to a transient shift from 5-HT$_7$ and 5-HT$_{2A}$ receptor-mediated neurotransmission to activation of 5- HT$_{1A}$ receptors (Beique et al. 2004). These results suggest coordinate changes in serotonergic regulation of cortical excitability at a time of active synaptogenesis.

In light of the evidence that serotonergic neurotransmission, mediated by 5-HT$_{2A}$ receptors, can regulate cerebral cortical activity during development, the present study sought to map 5-HT$_{2A}$ receptor protein expression and 5-HT and metabolite levels in the developing AC. The

purpose of this study was to identify critical periods during postnatal development when 5-HT, mediated by 5-HT_{2A} receptors, may play an integral role in establishing circuits in AC.

Materials and Methods

High Performance Liquid Chromatography

Sprague-Dawley rats (Charles River Laboratories) were used in these studies in accordance with the NIH Guide to the care and use of laboratory animals and institutional approval by The University of North Carolina at Chapel Hill Internal Animal Care and Use Committee. Rats (n=4 each at P10, 15, 20 and 28) were anesthetized with xylazine/ketamine (44 mg/kg–10 mg/kg) decapitated, and the forebrain removed. The brain was washed with ice-cold (4°C) dissection solution (in mM: 134 NaCl, 3.0 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1.25 KH_2PO_4, 10 glucose and 20 $NaHCO_3$; continually equilibrated with 95% O_2-5% CO_2, setting the pH to 7.3–7.4) and blocked to a region containing AC (Te1 and Te3) and adjacent tissues, and sliced (400 μm; Leica tissue slicer) in an oblique/horizontal orientation (Cruikshank et al. 2002). Sections at least 500 μm dorsal to the rhinal fissure were selected for study. Using a dissection microscope, AC was dissected away from each slice, placed in a microfuge tube and snap-frozen in liquid nitrogen. Each animal yielded 2 sections of isolated AC from each hemisphere. Samples were stored at −80°C until processed for high performance liquid chromatography (HPLC) with electrochemical detection, at which time they were homogenized in 300 μl ice-cold 0.1 M perchloric acid containing 1.0% ethanol, 0.02% EDTA and centrifuged for 30 min at 4°C. Supernatants (200 μl) from individual samples were then manually injected (Model 7125, Rheodyne Inc., Cotati, CA) onto a Bioanalytical Systems (BAS) HPLC system (West Lafayette, IN). Serotonin (5-HT) and metabolite 5-hydroxyindoleacetic acid (5-HIAA) were separated by a Biophase Phase II ODS column (3 μm 100 × 3.2 mm) at a mobile phase flow rate of 1.0 ml/min (PM-80 pump). The mobile phase consisted of 0.75 mM sodium phosphate, 0.5 mM EDTA, 1.4 mM octane sulfonic acid, and 3.4% acetonitrile and was adjusted to a final pH 3.0. Signals produced by monoamine oxidation were determined by LC-4C amperometric detectors (first electrode: −0.04 V; second electrode: +0.65 V) within each sample and were compared with signals of known concentration standards (Sigma Chemicals, St. Louis, MO). Final oxidation current values were expressed as picogram (pg) monoamine per mg tissue wet weight (mean ± SEM). Statistical analysis was performed using SPSS for Windows 7.0 and repeated with GraphPad Prism V5.0. For monoamine analyses, comparisons between postnatal groups were analyzed using a one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison *post hoc* test (significant at $p<0.05$).

5-HT_{2A} Receptor autoradiography

For radioligand binding studies, Sprague-Dawley rats (n=4 at P8, 10, 12, 17, 21, 28 and 35) were sacrificed under anesthesia as described above, and the brains removed and hemisected. Each hemisphere was snap-frozen in isopentane and stored at −80°Cuntil processed. Coronal brain sections (20μm) through the anterior-posterior extent of AC (Bregma: −3.80 to −4.80mm; Paxinos and Watson, 1986; which include both Te1 and Te3; Doron et al, 2002) were cut on a cryostat and thaw-mounted onto gelatin-coated slides and vacuum desiccated at 4°C overnight. Sections were incubated with ^{125}I- DOI ((+/−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; 200pM; 5-$HT_{2A/2C}$ receptor agonist) for 1 hour at RT in the presence of SB206553 (100nM; selective 5-HT_{2C} receptor antagonist to prevent DOI binding to 5-HT_{2C} receptors) in standard binding buffer (50mM TrisHCl, pH 7.4; 10mM MgCl, 0.1mM EDTA). Non-specific binding was determined by adding ritanserin (1μM; pan-5-HT_2 receptor antagonist) to the incubation mixture. Sections were washed (3 × 10 min) in ice-cold harvesting buffer (50mM TrisHCl, pH 7.4) air-dried and exposed to hyperfilm (GE Healthscience) along with ^{125}I-quantitation standards (Perkin-Elmer) for 6 days. Films were developed and photographed with a digital camera and analyzed (Metaview). Figure 2 demonstrates the area

of analysis (hash marks) dorsal to the rhinal fissure representing AC used for quantification. Using the ^{125}I-quantitation standards, tissue equivalent receptor densities (B) were calculated in nCi/mg protein, and converted to fmol/mg protein. Using the saturation binding equation ($B=B_{max}*[R]/(K_d + [R])$ and solving for B_{max}, 5-HT_{2A} receptor concentrations (for the high affinity state) were determined and expressed as fmol/mg protein (mean ± SD). Statistical analyses were performed using a non-parametric one-way analysis of variance (ANOVA, Kruskal-Wallis) followed by a Dunn's multiple comparison test (significant at $p<0.05$; GraphPad Prism V5.0). A non-parametric test was used as the data were not normally distributed. Prior to the ANOVA, extreme outliers in the data were removed (one point per animal, out of 10–70 measurements) using the Grubbs test (http://www.graphpad.com/quickcalcs/Grubbs1.cfm).

5-HT_{2A} Receptor immunocytochemistry

To specifically localize 5-HT_{2A} receptors within AC neurons, immunocytochemistry with confocal maging was conducted. Young adult (P35; n=4) Sprague-Dawley rats were transcardially perfused with 4% paraformaldehyde in 0.1M PBS. Brains were immersed in 4% paraformaldehyde overnight and then transferred to 0.1M PBS. Sections (50μm) through AC (Bregma: −3.80 to −4.80mm; Paxinos and Watson, 1986) were cut on a vibratome and stored in 0.1M PBS until processing for immunocyto-chemistry. Following multiple PBS washes (3 × 20 min), sections were pre-blocked (0.3% Triton X-100, 3.0% BSA and 7% normal goat serum, all in 0.1M PBS) for 2 hours at 4°C. Blocking buffer was removed and replaced with fresh buffer containing the anti-5-HT_{2A} receptor monoclonal antibody (PharMingen, San Diego, CA; 1:500) and incubated overnight at 4°C. To serve as a negative control, some sections were incubated in buffer with the primary antibody omitted (Fig. 4C). Sections were washed (3 × 20 min) in 0.1M PBS and incubated with goat-anti-mouse secondary antibody (Alexa; 1:1000) in blocking buffer for 2 hours at RT. Slides were washed (3 × 20 min) in 0.1M PBS and mounted on slides in aqueous mounting medium containing an anti-fading agent (Sigma). Qualitative images (Fig. 4) at high (40X objective) and low (10X objective) magnification were taken through the entire thickness of the auditory cortex in order to examine 5-HT_{2A} receptor protein expression by AC neurons.

Results

A1 Serotonin and metabolite levels

Fig. 1 graphically represents HPLC-derived tissue levels of 5-HT, 5-HIAA (5-hydroxyindoleacetic acid: Fig. 1A) and the 5-HT/5-HIAA ratio (Fig. 1B), indicative of 5-HT metabolism in AC during postnatal development. A 1-way ANOVA revealed a significant effect of age in both 5-HT ($p<0.05$) and 5-HIAA ($p<0.005$). Despite the significant effect of age, 5-HT levels in AC were not statistically significantly different between any of the ages. The serotonin metabolite, 5-HIAA, did not increase significantly between P10 and P15, but was significantly elevated to young adult levels at P20 and P28. Young adult levels at P28 were different from those at P10 ($p<0.05$), but not at other ages. Evaluation of the 5-HT/5-HIAA ratio, indicative of 5-HT metabolism, in AC also showed a significant effect of age (1-way ANOVA, $p<0.05$), such that it increased significantly between P10 and P20 ($p<0.05$). The 5-HT/5-HIAA ratio leveled off by P15 and did not increase significantly at P20 or P28 (Fig. 1B).

A1 5-HT_{2A} Receptor Binding

Figs. 2 and 3 show ^{125}I-labeled DOI binding indicative of 5-HT_{2A} receptor protein levels in the developing AC from P8-P35. Since DOI binds both 5-$HT_{2A/2C}$ receptors, sections were incubated with DOI following pre-incubation with the selective 5-HT_{2C} receptor antagonist, SB206553 to block binding to this receptor subtype. Fig. 2A shows representative

autoradiographs of coronal sections through the developing (P8–P28) rat brain with 5-HT_{2A} receptor binding in AC and adjacent cortical areas. Fig. 2B shows a higher magnification view at P35 to designate the specific area of analysis for quantification within AC. The left panel of Fig. 2B shows that the densest receptor binding is in layers II-III of AC, with lighter labeling in layer I and in layers IV through VI.

Figure 3 is a graphical representation of quantitative analysis of binding from sections such as those shown in Fig. 2. A 1-way ANOVA revealed a significant effect of age on receptor binding ($p<0.02$). Post-hoc tests showed that serotonin$_{2A}$ receptor levels significantly increased between P8 and P10 ($p=0.02$), did not significantly increase at P12, but were significantly elevated at P17 ($p<0.05$), then fell to young adult levels by P35. At P21-35, the receptor levels were not significantly different than at P8. These data demonstrate a developmental increase in postnatal 5-HT_{2A} receptor protein levels, which peak during the second and third weeks of postnatal development, then decreases by P35.

A1 5-HT_{2A} Receptor Immunocytochemistry

The radioligand receptor binding experiments had limited spatial resolution, preventing us from clearly identifying the cellular localization of 5-HT_{2A} receptors. Consequently, we used confocal microscopy to clarify the cellular elements and the 5-HT_{2A} receptor distribution across layers of AC, using immunocytochemical labeling of 5-HT_{2A} receptors with a specific monoclonal. Fig. 4 shows confocal images of AC neurons expressing 5-HT_{2A} receptor immunoreactivity (5-HT_{2A} IR) at low and high magnification. Low magnification evaluation (Fig. 4A) demonstrates robust expression of 5-HT_{2A} receptors by neurons across all layers of AC, although the densest labeling is in layers II/III. Higher magnification (Fig. 4B) demonstrates 5-HT_{2A} IR within cell bodies and apical dendrites of pyramidal neurons in layers II/III of the ventral AC. Figure 4C shows the absence of labeling in the negative control, where primary antibody was omitted from the assay. These images suggest that binding of ^{125}I- DOI to 5-HT_{2A} receptors is likely confined to cell bodies and apical dendrites of layer II/III pyramidal neurons.

Discussion

The present study represents the first evaluation of 5-HT_{2A} receptor levels and 5-HT metabolite content specifically in the developing rat AC. The results clearly demonstrate a developmental dynamic for 5-HT_{2A} receptors and the 5-HT/5-HIAA ratio, which progressively increase during the second and third weeks of postnatal AC development. This may reflect important spatio-temporal relationships between 5-HT axons and 5-HT_{2A} receptors expressed by developing AC neurons, and may have implications for normal maturation of auditory functioning and processing.

Serotonin and 5-HIAA levels in AC both gradually increased during the second and third postnatal weeks to reach adult levels by P20, whereas the 5-HT/5-HIAA ratio, indicative of serotonergic neurotransmission and metabolism, already reached adult levels by P15. This correlates with the robust ontogeny of 5-HT projections to the cerebral cortex between E21 and P21 (Lidov and Molliver 1982; Wallace and Lauder, 1983), a critical period of rapid axonal and dendritic growth, and synaptogenesis, between P6–P18 (Blue and Parnavelas 1983a; Blue and Parnavelas 1983b; Micheva and Beaulieu 1996; Miller and Peters 1981; O'Leary et al. 1994; Stern et al. 2001; Uylings et al. 1993). These events culminate in the concurrent differentiation of neurons and the formation of cortical layers (Rice et al. 1985; Van Eden and Uylings 1985). The possibility that 5-HT may be involved in normal postnatal AC development is supported by the findings that (1) early 5-HT depletion delays the onset of differentiation (time of last cell division) in brain areas receiving 5-HT afferents while progenitors are still dividing (Lauder and Krebs 1978; Lauder et al., 1982), and (2) alterations in cortical

serotonergic innervation during early postnatal development disrupts the formation of barrel-fields in layer IV of the somatosensory cortex (Bennett-Clarke et al. 1994c; Cases et al. 1996; reviewed by Luo et al., 2003).

The exact role that 5-HT projections and receptors play in AC development and ultimate functioning is not completely clear. Most 5-HT studies have focused largely on the frontal cortex, and have shown that as serotonergic axons from the dorsal and median raphe innervate the frontal cortex (Azmitia and Segal 1978; Conrad et al. 1974; Dori et al., 1996; Lidov and Molliver 1982; Wallace and Lauder, 1983; Wilson and Molliver 1991), 5-HT receptors are developing there (D'Amato et al., 1987; Hellendall et al., 1993; Martin-Ruiz et al. 2001), including 5-HT_{2A} receptors, which are localized to pyramidal cells and interneurons (Willins et al. 1997). Confocal microscopy from the present study demonstrates robust 5-HT_{2A} receptor IR in AC pyramidal cell bodies and pyramidal cell apical dendrites in young adult (P35) rats, in accord with previous data using the same 5-HT_{2A} receptor antibody (Li et al., 2004). Serotonin$_{2A}$ receptor binding in AC rose sharply from P8 to P10, peaked at about P17 and tapered to young adult levels thereafter (Fig. 3). This is consistent with previous receptor binding studies using ^{3}[H] ketanserin in the developing rodent brain, which reported an 8-fold increase in 5-HT_2 receptors between embryonic day E17 and P13, and a 13-fold increase in 5-HT_2 receptor mRNA between E17-P5 (Roth et al. 1991). Interestingly, while increases in 5-HT_{2A} receptors during the second postnatal week may reflect a temporal relationship between 5-HT axons and AC cortical neurons (Lidov and Molliver 1982), the 5-HT neurotoxin, 5,7-DHT, does not alter 5-HT_{2A} receptor mRNA expression in neonatal rodents (Basura and Walker, 1999), nor 5-HT_2 receptor binding in adults (Fischette et al. 1987), suggesting that 5-HT_2 receptor biosynthesis may be independent of the influence of developing 5-HT innervation. While there is no direct evidence for a decline in 5-HT_{2A} receptors in layer V pyramidal cells of the adult cortex (Cornea-Hebert et al., 1999), the function of 5-HT_{2A} receptors, as measured by phosphoinositide turnover, is maximal early in postnatal development, and decreases in the adult rat brain (Claustre et al. 1988; Ike et al. 1995). This is consistent with results of the present study, which show that the 5-HT/5-HIAA ratio, indicative of 5-HT metabolism, peaks to young adult levels by the second postnatal week, then levels off thereafter. Taken together, these data suggest that anatomically and temporally, 5-HT_{2A} receptors and 5-HT projections have similar developmental time courses during the critical period of AC development. This may have implications for the establishment of auditory circuitry.

From a functional standpoint, the exact role of early 5-HT innervation and receptor linkage in AC neurons is also unclear. Serotonin neurotransmission, via 5-HT_{1A} receptors mediates an inhibitory hyperpolarization, whereas 5-HT_{2A} receptors mediate depolarization in pyramidal cell neurons of the cerebral cortex in juvenile rats and guinea pigs (Araneda and Andrade 1991; Davies et al. 1987; Tanaka and North 1993). Stimulation of 5-HT_{2A} receptors has been shown to induce tonic firing of layer V pyramidal neurons in frontal cortex during early postnatal development (Zhang 2003), through a postsynaptic mechanism that decreases greatly after P14, such that by P21, 5-HT produces little effect on membrane potential in these neurons. In contrast, 5-HT continues to induce large depolarizations and cell firing in the brainstem and hypothalamus of young adult (P20–P30) or adult rats (Eriksson et al. 2001; Talley et al. 1997), while 5-HT, via 5-HT_2 receptors, can prolong bursts of spontaneous inhibitory postsynaptic currents in the lateral superior olive; an effect rarely observed beyond P8 (Fitzgerald and Sanes, 1999). Moreover, Juckel and colleagues (1999), demonstrated that inhibition of cat dorsal raphe 5-HT neurons by the 5-HT_{1A} receptor agonist, 8-OH-DPAT, increased the intensity of AC evoked bpotentials, while spiperone (a mixed 5-HT_{1A}/5-HT_{2A} antagonist; Nogueira and Graeff, 1995), decreased these potentials. While Juckel et al (1999) did not examine the developmental time course of the 5-HT modulation in AC, these findings, taken together with our study, suggests a possible role for 5-HT and 5-HTreceptor

regulation of AC neuronal activity during the first weeks after the onset of hearing. Consequently, 5-HT neurotransmission, mediated by multiple receptor subtypes, may inhibit or excite neurons at different stages of development. This functional linkage may change during early postnatal development, and be modulated by sensory experience to shape different critical periods for the acquisition of sensory processing (Hegerl and Juckel 1993; Kahkonen et al. 2002a; Kahkonen et al. 2002b).

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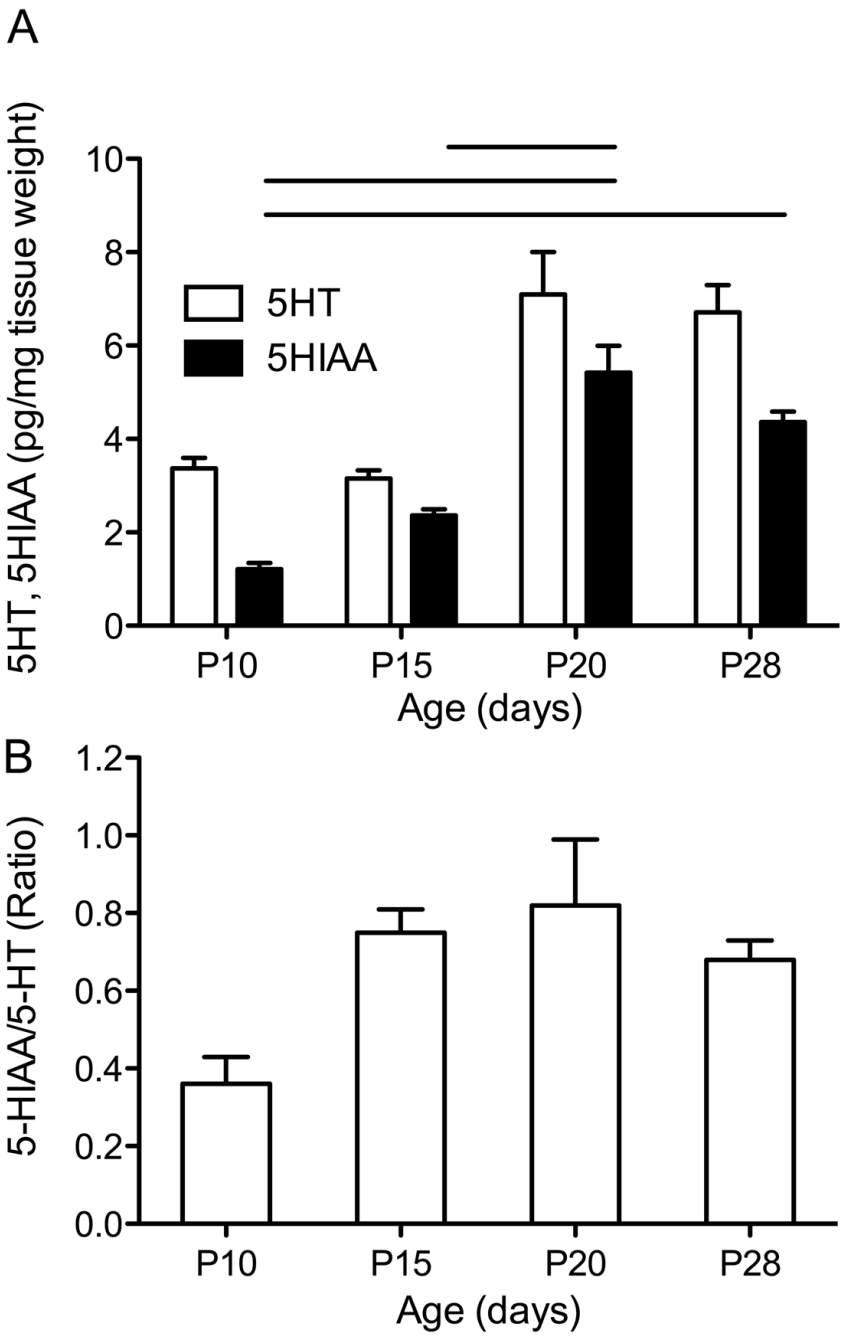

Figure 1.

5-HT and 5-HIAA tissue levels in developing AC. **A.** Relative levels of 5-HT and 5-HIAA expressed as picograms of monoamine per mg of tissue weight in normal postnatal AC development. Despite a significant effect of age, no individual 5-HT age comparisons were significantly different. ANOVA followed by a Tukey's multiple comparison test shows that 5-HIAA levels were significantly different at P10 vs P20 ($p<0.01$); P10 vs P28 ($p<0.05$) and P15 vs P20 ($p<0.05$). **B.** 5-HT/5-HIAA ratio indicative of 5-HT metabolism in AC during normal development. Young adult (P28) ratio is reached by P15. The only significantly different levels are found between P10 and P20 ($p<0.05$). The lines above the bar graph link significantly different comparisons.

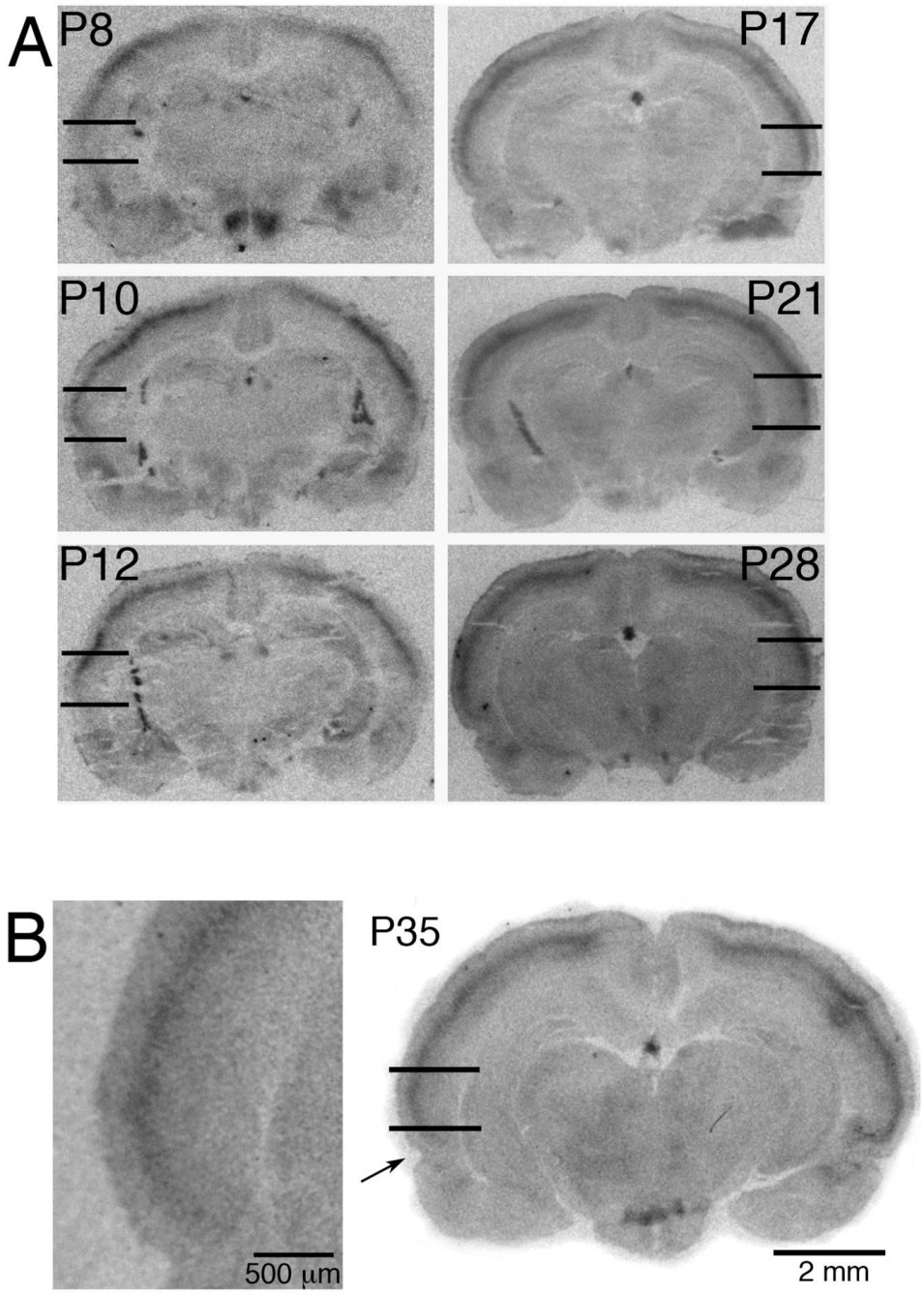

Figure 2.

A. Whole brain autoradiograph images demonstrating ^{125}I-DOI labeling of 5-HT$_{2A}$ receptors within the rat AC during postnatal development from P8 to P35. Dark bars represent the limits of AC (including Te1 and Te3) and the regional of analysis. **B.** Right: Enlarged image of P35 rat brain demonstrating area of analysis (bars) representing AC used for quantification in each section. Note the robust signal dorsal to the rhinal fissure (arrow) extending from AC to other cortical areas. Left: Higher magnification shows that the 5-HT$_{2A}$ receptor binding in AC is highest in layers II and III, and lower in other layers. This anatomic location was confirmed with immunocytochemistry and confocal microscopy (see Figure 4).

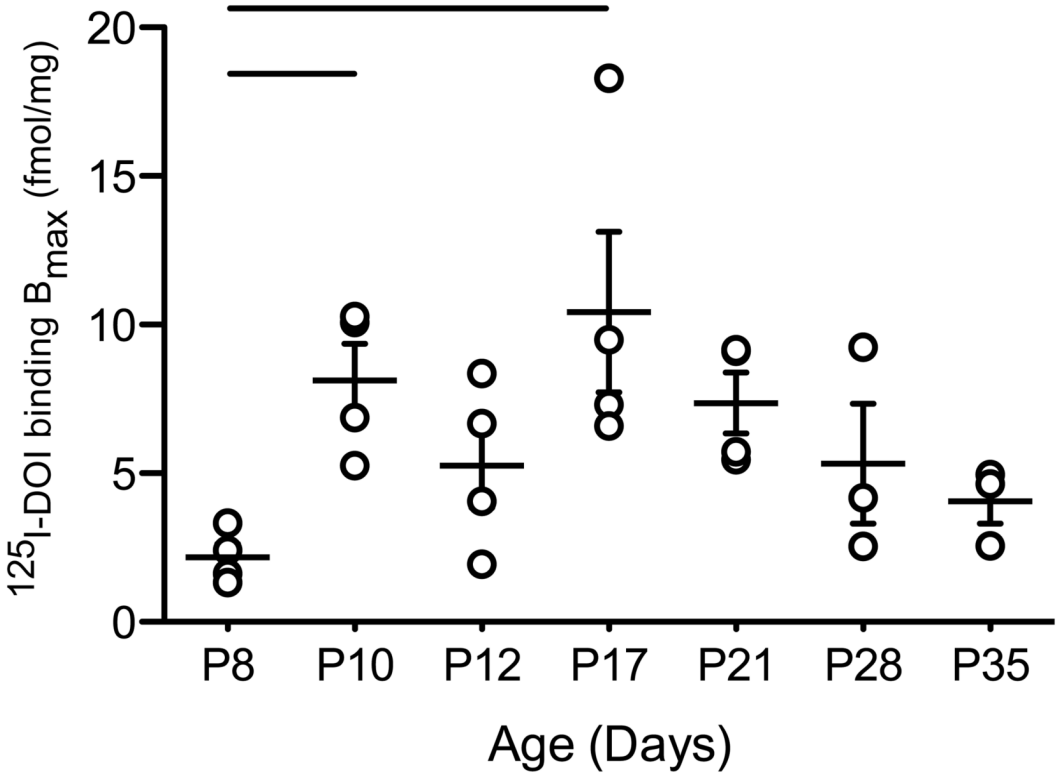

Figure 3.

Quantified ^{125}I-DOI radio-ligand binding of 5-HT_{2A} receptors within the developing AC expressed as fmol/mg. The graph represents pixel densities, converted to tissue binding levels, from radio-ligand binding of 5- HT_{2A} receptors from P8 to P35 (autoradiographs shown in Fig. 2). Data are shown as the individual measurements from each animal (open circles), and the mean and SEM for each age. Statistical analysis was performed using a non-parametric one-way ANOVA (Kruskal-Wallis, significance at $p<0.05$). 5-HT_{2A} receptor expression increases to young adult levels within the first two weeks of postnatal development, and declines thereafter. Post-hoc tests (Dunn's) showed that significant increases were seen between P8 and P10 and P8 and P17. There was no difference between P8 and measurements at P21, P28 and P35, suggesting a significant decline following P17. The line above the bar graph links significantly different comparisons.

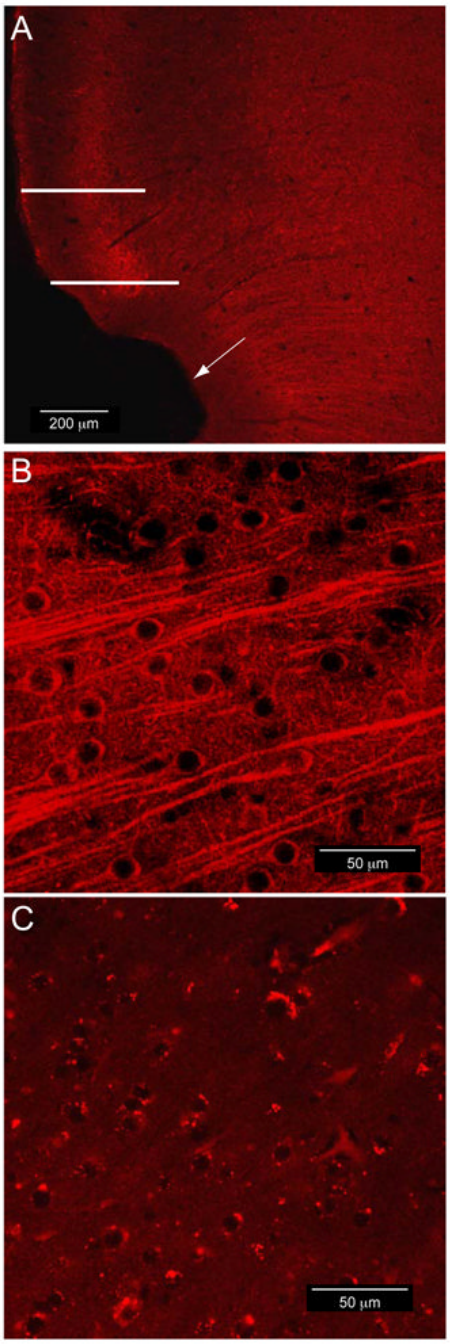

Figure 4.

5-HT_{2A} receptor expression in AC using immunocytochemistry and confocal imaging. **A.** Low power image (10X objective) showing the position of the rhinal fissure (arrow) and dorsally located 5-HT_{2A} receptor immunoreactive dendrites within the AC using a 5-HT_{2A} receptor monoclonal antibody (PharMingen; 1:500; note: bars outline AC region containing Te1 and Te3 seen at higher magnification in panels B and C). **B.** High magnification (40X objective) from panel A showing 5-HT_{2A} receptor immunoreactivity within apical dendrites of pyramidal neurons located within layers II/III of the AC. **C.** Negative control section in which the primary 5- HT_{2A} receptor antibody was omitted from the assay. Note background staining but no labelling of cell bodies or fibers.