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Development of Brainstem 5-HT_{1A} Receptor Binding Sites in Serotonin-Deficient Mice

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Abstract

The sudden infant death syndrome (SIDS) is associated with a reduction in brainstem serotonin (5-hydroxytryptamine, 5-HT) and 5-HT_{1A} receptor binding, yet it is unknown if and how these findings are linked. In this study, we used quantitative tissue autoradiography to determine if postnatal development of brainstem 5-HT_{1A} receptors is altered in two mouse models where the development of 5-HT neurons is defective, the *Lmx1b^{f/f/p}*, and the *Pet-1^{-/-}* mouse. 5-HT_{1A} receptor agonist-binding sites were examined in both 5-HT-source nuclei (autoreceptors) and in sites that receive 5-HT innervation (heteroreceptors). In control mice between postnatal day (P) 3 and 10, 5-HT_{1A} receptor binding increased in several brainstem sites; by P25, there were region-specific increases and decreases, refining the overall binding pattern. In the *Lmx1b^{f/f/p}* and *Pet-1^{-/-}* mice, 5-HT_{1A}-autoreceptor binding was significantly lower than in control mice at P3, and remained low

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at P10 and P25. In contrast, 5-HT_{1A} heteroreceptor levels were comparable between control and 5-HT-deficient mice. These data define the postnatal development of 5-HT_{1A} receptor binding in the mouse brainstem. Furthermore the data suggest that 5-HT_{1A} heteroreceptor deficits detected in SIDS are not a direct consequence of a 5-HT neuron dysfunction nor reduced brain 5-HT levels.

Keywords

sudden infant death syndrome; autoreceptors; heteroreceptors; raphe; Pet1; Lmx1b

Introduction

Serotonin (5-hydroxytryptamine, 5-HT) is a major monoamine neurotransmitter that communicates with multiple receptor subtypes throughout the neuroaxis to regulate key physiological and behavioral functions. Importantly, it helps regulate brainstem respiratory and autonomic responses to homeostatic challenges, in part by virtue of actions at the 5-HT_{1A} receptor (Darnall *et al.* 2005, Audero *et al.* 2008, Pham-Le *et al.* 2011, Curran & Leiter 2007). 5-HT_{1A} receptors are located somatodendritically on 5-HT neurons, where they function as autoreceptors. In addition, 5-HT_{1A} receptors are located on non-5-HT neurons and act as heteroreceptors in regions of the brainstem and forebrain that receive 5-HT innervation. Autoreceptor and heteroreceptor populations are known to exhibit region-specific adaptations to the over- or under-availability of ligand (reviewed by (Hensler 2003)).

Alterations in brainstem 5-HT_{1A} receptors are associated with several human disorders (Kinney *et al.* 2011) including the sudden infant death syndrome (SIDS) (Waters 2010, Panigrahy *et al.* 2000, Paterson *et al.* 2006, Duncan *et al.* 2010, Saito *et al.* 1999, Ozawa & Takashima 2002). The leading cause of postneonatal mortality in the United States today, SIDS is defined by sleep-related death in the first postnatal year of life that is unexplained by a complete autopsy and death scene investigation (Kinney & Thach 2009). The majority of SIDS deaths are associated with asphyxia-generating circumstances that appear to trigger death, e.g., rebreathing exhaled gases in the face-down (prone) sleep position (Pasquale-Styles *et al.* 2007, Kinney & Thach 2009). Accordingly, a leading hypothesis in SIDS research today is that SIDS is due to a brainstem abnormality that impairs the ability to generate protective responses to life-threatening challenges (Kinney & Thach 2009, Kinney *et al.* 2009). Indeed 5-HT_{1A} receptors reductions have been observed in the brainstem of SIDS infants that involve both auto- and heteroreceptor populations (Kinney *et al.* 2003, Duncan *et al.* 2010, Paterson *et al.* 2006, Machaalani *et al.* 2009). Lower 5-HT_{1A} receptor binding in SIDS cases is associated with decreased medullary tissue content of 5-HT and tryptophan hydroxylase 2 (TPH2), the rate-limiting biosynthetic enzyme for 5-HT, suggesting a 5-HT-deficient disorder (Duncan *et al.* 2010).

Two murine models have been instrumental in examining the developmental role of 5-HT on cardiorespiratory function and thermoregulation, i.e., homeostatic processes potentially relevant to the pathogenesis of SIDS (Richerson & Buchanan 2011, Buchanan & Richerson 2010, Hodges *et al.* 2009, Cummings *et al.* 2011a, Kinney & Thach 2009, Erickson *et al.*

2007). These 5-HT deficient models include a conditional removal of the transcription factor *Lmx1b* selectively in *Pet1*-expressing cells (*Lmx1b^{flox/flox};ePet-cre*) resulting in the '*Lmx1b^{ff/p}*' mouse in which there is nearly a complete loss of 5-HT neurons by midgestation and consequently 5-HT in the central nervous system (Zhao *et al.* 2006). The other model is the *Pet-1^{-/-}* mouse in which 5-HT neurons persist in the brain but the majority fail to differentiate appropriately and do not produce 5-HT (Cummings *et al.* 2011a, Hendricks *et al.* 2003, Erickson *et al.* 2007). Detectable 5-HT synthesis appears to occur in only about 30% of the normal number of 5-HT neurons, and these residual neurons innervate a selective subset of brain regions, predominantly regions associated with stress responses (Kiyasova *et al.* 2011).

In this study, we examined how 5-HT_{1A} receptors binding patterns develop in the brainstem in these two mouse models with 5-HT deficiency. We applied the methodology of quantitative tissue autoradiography, a technique used to study SIDS tissue, in part to allow for comparisons to 5-HT_{1A} receptor binding in human brainstem disorders (Paterson *et al.* 2006). We tested the hypothesis that decreases in 5-HT_{1A} heteroreceptor binding in the brainstem during development may be caused by 5-HT neuron dysfunction or associated 5-HT deficiency. Given the absence of 5-HT neurons in the *Lmx1b^{ff/p}* mouse, and the demonstrated loss of 5-HT_{1A} receptor gene expression in the dorsal and median raphe nuclei of *Pet-1^{-/-}* mice (Liu *et al.* 2010, Jacobsen *et al.* 2011), we also anticipated finding a large decrease in medullary 5-HT_{1A} autoreceptors. As tissue autoradiography does not reveal the cellular location of receptors, we operationally defined 5-HT_{1A} autoreceptors as those within the 5-HT source nuclei and 5-HT_{1A} heteroreceptors as those localized to nuclei receiving 5-HT projections.

2. Materials and methods

2.1 Animals

Two different mouse strains were used in this experiment: *Lmx1b* conditional knockout in *Pet1*-expressing cells (*Lmx1b^{flox/flox};ePet-Cre/+* (*Lmx1b^{ff/p}*)) and *Pet-1^{-/-}* mice. *Lmx1b^{ff/p}* mice were bred at Yale University in New Haven, Connecticut, and *Pet-1^{-/-}* mice at the Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire. Animal protocols were approved by the Institutional Animal Care and Use Committees at these institutions, and were consistent with guidelines of the National Institutes of Health. For *Lmx1b^{ff/p}* mice, controls were siblings lacking Cre recombinase on a C57BL/6 background. For *Pet-1^{-/-}* mice, controls were heterozygote and control siblings from a mixed C57BL/6 and 129 background; heterozygotes have been shown to have normal number of 5-HT immunolabeled neurons (Hendricks *et al.* 2003)Cummings *et al.* 2011a). Both male and female mice were included and were analyzed at P3, P8-10 and P25. These ages were selected based upon the severe respiratory deficits in *Lmx1b^{ff/p}* mice at neonatal (<P4) ages (Hodges *et al.* 2008) and vulnerability of *Pet-1^{-/-}* mice to repeated hypoxic challenge at P8/10 (Cummings *et al.* 2011a). Group size for the P3 and P8-10 groups averaged 7.5 and 7 individuals respectively, with a minimum of 5 per group; for the P25 age groups the average group size was 4.75 individuals, with a minimum of 4 individuals per group.

2.2 Tissue Collection

Under deep anesthesia, mouse brains were removed, frozen and stored at -80° C until sectioned 20 µm thick with a cryostat. Sections were collected serially onto sets of 4 slides such that each slide contained every fourth section. Slides were stored at -80°C until processed for autoradiography.

2.3 Autoradiography

Slides were gradually warmed to room temperature before tissue incubations for autoradiography, using a protocol adapted from human studies (Paterson *et al.* 2006, Duncan *et al.* 2010). One quarter of the slides collected for each mouse was used for total radioligand binding, while another quarter containing the adjacent sections was processed to determine non-specific binding levels. Buffer solution consisted of 0.17 M Tris with 0.01% ascorbic acid and 4mM CaCl₂ (pH 7.6). At room temperature, slides were preincubated in buffer solution for 30 minutes. Then, sections were incubated in 4nM ³H-8-hydroxy-2-(di-n-propylamino)tetralin (DPAT) and 10 µM pargyline diluted in fresh buffer solution for one hour for total specific binding. For nonspecific binding, 10 µM 5-HT was added to the DPAT-containing solution, and slides were incubated for one hour. Incubated slides were washed in ice-cold incubation buffer solution twice for five minutes. All sections were then air dried for 15 min using a cold air stream, and then placed on a slide warmer for 30 minutes to dry completely.

2.4 Quantitative Data analysis

Slides for total and non-specific binding were exposed on ³H -sensitive film with one ³H -standard slide (Amersham) for 3 weeks. For quantification of receptor binding density, NIH Image J software was used. In order to convert optical density into femtomoles/milligram tissue (fmol/mg), a calibration curve was generated using the ³H-standards. Seven sites were selected for analysis (Fig. 1) that were representative of auto- and heteroreceptor sites: 3 nuclei that contain 5-HT source neurons (raphe obscurus, dorsal raphe, and lateral paragigantocellular nucleus [LPGi]), and 4 sites that receive 5-HT projections (spinal trigeminal nucleus, inferior colliculus, interpeduncular nucleus, and cerebellum). At least in adults, the majority of 5-HT_{1A} receptors co-localize with markers of serotonin neurons in the raphe nuclei, thus we infer these areas largely represent autoreceptor populations (Czesak *et al.* 2012). For each region, 5-HT_{1A} receptor binding density was measured in three sections/mouse and the values averaged to determine total and non-specific binding. Non-specific binding values were subtracted from those of total binding to obtain specific binding. Subsequently, individual values were averaged to yield group means and standard error of the mean.

Statistical Analysis

For each mutant group, data were first analyzed using gender as a factor. When there was no significant effect of gender, males and females were pooled and analyzed together. The data were compared using a three-factor ANOVA for age (at three levels), genotype (at two levels), and region, with region as a repeated measure. Following a significant main effect of region, each region was analyzed independently using slice ANOVAs for age and genotype

with Tukey or Student's-t post hoc tests, depending on the number of comparison groups. Mutant groups were not directly comparable to each other because of differences in genetic background, rearing location, and ages at harvesting, which were associated with some modest differences between the two control groups in 5-HT_{1A} receptor binding. Therefore, to evaluate the relative loss of receptor binding in each mutant line (Fig. 5), the binding values were normalized to the appropriate control values yielding a 'percent retained binding' value (i.e. mutant binding/mean control binding expressed as a percent). These values were averaged, and a standard error was determined for each genotype and age. 5-HT_{1A} receptor-binding deficiencies in each mutant in the dorsal raphe nucleus and the raphe obscurus were then compared using ANOVA followed by Student's t-test.

Results

Age Effects

In control mice at P3, the dorsal raphe, inferior colliculus, and spinal trigeminal nucleus had the highest levels of 5-HT_{1A} binding (greater than 30 fmol/mg) (Fig. 1, 2, 3). Intermediate binding, in the range of 20-30 fmol/mg, was found in the raphe obscurus and LPGi, and low binding (<20 fmol/mg) in the remaining sites analyzed, including the cerebellum. Between p3 and P10, in every site measured except the cerebellum, there were significant increases in 5-HT_{1A} -receptor binding (Fig 1, 2). Between P10 and P25, control mice showed region-dependent changes in 5-HT_{1A} -receptor binding levels (Fig. 1, 3). Two sites exhibited further increases in binding, including the interpeduncular nucleus and the dorsal raphe nucleus. In four sites, the inferior colliculus, LPGi, spinal trigeminal nucleus and raphe obscurus, there were decreases in 5-HT_{1A} receptor binding. These patterns of developmental changes in the control mice for *Lmx1b^{ff/p}* were the same in the *Pet-1^{-/-}* control group (Fig. 4).

Genotype Effects, *Lmx1b^{ff/p}* mice

At P3, in comparison to control mice, *Lmx1b^{ff/p}* mice showed significantly reduced binding only in two sites, the dorsal raphe nucleus and raphe obscurus (Fig. 1, 2, 3), i.e., major sites of 5-HT source neurons in the brainstem. At P10, 5-HT_{1A} binding was reduced in the dorsal raphe, inferior colliculus and raphe obscurus in mutants compared to their controls (Fig. 3). At P25, the magnitude of the reduction of 5-HT_{1A} binding in the dorsal raphe nucleus and raphe obscurus was severe, with binding in the *Lmx1b^{ff/p}* more than two-thirds reduced from control values (Fig. 3). The maintenance of heteroreceptor binding in the quantified areas appeared representative of additional heteroreceptor populations, such as in the nucleus of the solitary tract (Fig. 2).

Genotype Effects, *Pet-1^{-/-}*

In *Pet-1^{-/-}* mice at P3, the only site with a significant reduction compared to control was in the dorsal raphe nucleus (Fig. 4). At P8 both the dorsal raphe and raphe obscurus, however, demonstrated significant reductions in binding compared to controls. At P25, the additional site of the LPGi in *Pet-1^{-/-}* mice had significantly less binding than control values (Fig. 4). In comparison to *Lmx1b^{ff/p}* mice (Fig. 5), the loss of 5-HT_{1A} binding in the dorsal raphe nucleus and raphe obscurus was comparable at P3 and P8/P10, however, at P25 5-HT_{1A}

binding levels in *Pet-1*^{-/-} mice were half that seen in their controls, a less severe loss than that seen in *Lmx1b*^{f/f/p} mice.

Discussion

Previous studies have reported an association between altered brainstem 5-HT neurochemistry and SIDS. Specifically, there is evidence for aberrant 5-HT neuron function including decreases in tryptophan hydroxylase 2 and tissue 5-HT content (Duncan *et al.* 2010). In addition, there are consistent reports of decreases in 5-HT_{1A} receptor binding (Duncan *et al.* 2010, Paterson *et al.* 2006, Machaalani *et al.* 2009). This not only involves 5-HT_{1A} autoreceptors, but also 5-HT_{1A} heteroreceptors in multiple medullary sites. The question remains as to whether 5-HT_{1A} heteroreceptor deficits can be explained by a primary dysfunction in 5-HT neurons themselves. In this study we examined the postnatal development of 5-HT_{1A} receptors in two mouse models with genetically-induced deficits in 5-HT neuron function. Our data indicate a decrease in 5-HT_{1A} binding in both mouse strains in areas that are normally enriched with 5-HT neurons (autoreceptors), confirming and extending previous observations to younger ages and medullary sites (Liu *et al.* 2010, Jacobsen *et al.* 2011). However, heteroreceptors, or 5-HT_{1A} receptors located in areas containing non-5HT neurons, were largely comparable to control values in the brainstem at all postnatal ages examined.

Methodological Considerations

5-HT_{1A} -receptor autoradiography is a classic approach used to quantify binding sites in the brain. Strengths of this approach include the ability to compare results to studies in human pathology cases, such as those employed in the study of SIDS. In addition, many studies using animal models have examined changes of 5-HT_{1A} receptor levels in response to manipulations of the 5-HT system or to drug exposure, particularly in the case of serotonin-selective reuptake inhibitors (Rossi *et al.* 2008, Hensler 2002, Hensler *et al.* 1991, Welner *et al.* 1989). However, it should be noted that there are potential adaptations in 5-HT_{1A} -receptor function, including changes in the ability to activate G-proteins and subsequent intracellular signaling cascades that are not detected using receptor autoradiography, and require alternative methods to evaluate (Fairchild *et al.* 2003, Hensler 2003). Indeed, receptor autoradiography provides view of 5-HT_{1A} receptors that is distinct and complimentary to methods that assay protein, mRNA levels, or intracellular signaling.

In both 5-HT-deficient mutant mouse lines, there were decreases in 5-HT_{1A} autoreceptor levels. These reductions are likely a direct consequence of the relevant genetic mutations and associated cell loss or transfating rather than a compensatory change in response to the loss of the endogenous ligand, 5-HT. In *Lmx1b*^{f/f/p} mice, the loss of *Pet1* expression and indeed, 5-HT neurons themselves would be expected to produce a parallel loss of 5-HT_{1A} autoreceptors (Ding *et al.* 2003, Zhao *et al.* 2006). Any residual 5-HT_{1A} receptor binding in the raphe nuclei of *Lmx1b*^{f/f/p} mice could be associated with non-5-HT neurons or glia. With respect to *Pet-1*^{-/-} mice, loss of autoreceptor binding in the *Pet-1*^{-/-} mice is consistent with previously detected decreases in mRNA expression in the dorsal raphe nucleus and loss of agonist-evoked effects on currents (Jacobsen *et al.* 2011, Liu *et al.* 2010). Indeed, in 5-HT

neurons *Pet1* is a direct transcriptional enhancer of the *5-HT_{1A} receptor* gene, while *5-HT_{1A} receptor* transcription in non-5-HT neurons is *Pet1*-independent (Jacobsen *et al.* 2011). Therefore, lack of transcriptional activation of the *5-HT_{1A} receptor* gene would be expected to result in a lower level of 5-HT_{1A} receptor expression in *Pet1*-dependent 5-HT neurons. However, in the *Pet-1^{-/-}* mice there is a residual population of 5-HT neurons that appear *Pet1*-independent, and these neurons could potentially express 5-HT_{1A} receptors independent of *Pet1* function (Hendricks *et al.* 2003, Kiyasova *et al.* 2011). Alternatively, a very low level of expression could persist in residual neurons within the area. These explanations could account for the greater magnitude loss of 5-HT_{1A} receptor binding in the *Lmx1b^{ff/p}* mouse compared to the *Pet-1^{-/-}* mouse detected at P25.

Loss of the endogenous ligand, 5-HT, in both of these mutants does not appear to consistently impact 5-HT_{1A} -heteroreceptor binding levels in brainstem projection sites during postnatal development. Adaptations in 5-HT_{1A} receptor binding in response to manipulations that increase or decrease available 5-HT have primarily been studied in the adult forebrain and in the primary sources of forebrain 5-HT, the dorsal and median raphe nuclei (reviewed by (Frazer & Hensler 1990, Hensler 2003)). These studies show that changes in 5-HT_{1A} receptors can be complex and may not only involve changes in receptor number or distribution, but also in signaling capacity, and furthermore, the specific changes that occur appear to be region-dependent (Hensler 2003). In the most relevant example, the adult *Pet-1^{-/-}* mouse using Western blot, compensatory *increases* of 5-HT_{1A} receptors were detected in the hippocampus while *decreases* were found in the striatum (Jacobsen *et al.* 2011). Increases in hippocampal 5-HT_{1A} receptors are also found in cases where 5-HT projections were chemically lesioned, or 5-HT_{1A} receptor signaling was blocked by administration of an antagonist (Patel *et al.* 1996, Abbas *et al.* 2007). Our results would extend these observations using genetic models with constitutive developmental losses of 5-HT and show that at several brainstem sites no changes in 5-HT_{1A} -receptor binding are detected. Likewise the results confirm that transcriptional control of 5-HT_{1A} receptors is region dependent in that heteroreceptors throughout the brainstem is *Pet-1*-independent, as indeed, *Pet1* expression is restricted to serotonin neurons (Albert *et al.* 2011, Jacobsen *et al.* 2011, Hendricks *et al.* 1999, Pfaar *et al.* 2002).

The maintenance of 5-HT_{1A} heteroreceptors in the mutant mouse lines also suggests that 5-HT_{1A} heteroreceptor levels are unaffected by the severe physiological defects displayed in these mouse lines. Specifically, both mouse lines have lower weight gain during the first few weeks of life. In addition, respiratory defects in both lines are likely to impact brain oxygenation and pH: as neonates, *Lmx1b^{ff/p}* have dramatic and prolonged (1-2 min) spontaneous apneas (Hodges *et al.* 2009), and as adults, both *Lmx1b^{ff/p}* and *Pet-1^{-/-}* have abnormally low respiratory responses to CO₂ (Hodges & Richerson 2008, Hodges *et al.* 2011). Finally, both lines are severely compromised in their ability to thermoregulate during environmental cooling (Cummings *et al.* 2011b, Hodges *et al.* 2011, Hodges & Richerson 2010). While these effects are interpreted as consequences of the altered state of 5-HT neurons, the current results provide evidence that these physiological deficits do not independently impact 5-HT_{1A} heteroreceptor binding in these mice.

This study further describes the developmental timecourse of 5-HT_{1A} receptor binding in the brainstem during the early postnatal period in mice. Analysis of the 5-HT_{1A} binding sites in several brainstem areas revealed two phases of development in the mouse. Before P8-10 there are broad increases in 5-HT_{1A} -receptor binding. After P8-10, there are region-dependent increases or decreases that serve to refine the overall pattern of 5-HT_{1A} -receptor binding. Consistent with the time-course of changes observed, the first three postnatal weeks are known to constitute an important period of synaptic development and refinement in mouse (Bhatt *et al.* 2009). Indeed, P8, an age of apparent rapid changes in 5-HT_{1A} receptor binding, corresponds to an age of peak vulnerability to repeated hypoxic stress in *Pet-1*^{-/-} mice (Cummings *et al.* 2011a). Likewise, an extended postnatal development of 5-HT_{1A} receptor-binding sites has been described in the forebrain (Miquel *et al.* 1994). In the rat hypoglossal nucleus, a similar biphasic development of 5-HT_{1A} receptors is observed where mRNA and binding increase until day 7 and decrease thereafter (Talley *et al.* 1997). However, it should be noted that our results differ from those of (Liu & Wong-Riley 2010) who showed with immunohistochemistry in rat that 5-HT_{1A} receptor levels in several brainstem nuclei are high at birth, only decreasing after two weeks of age. These differences may be related to species differences or the different methodology employed.

Conclusion

Our data suggest that the decreased 5-HT_{1A} heteroreceptor binding in medullary regions of SIDS cases may not be caused by dysfunction of 5-HT neurons nor the associated reduced brain 5-HT levels. 5-HT_{1A} receptors are known to be regulated by multiple transcriptional enhancer and repressors, including NF-kappa-B, Freud, REST, Deaf1, Hes proteins and glucocorticoid receptors (Albert *et al.* 2011). Future studies may hone in on the mechanism of dysregulation of 5-HT receptors relevant for SIDS. Importantly it should be noted that in these mutants there is a profound loss of ligand, thus although present, 5-HT_{1A} heteroreceptors may not endogenously function as normal. Therefore these mouse lines continue to be useful to understand the importance of 5-HT to homeostatic functions relevant to SIDS. The results also reveal that the postnatal period in mouse development is associated with rapid and dramatic changes of 5-HT_{1A} receptor binding that may be relevant to age-dependent disorders in humans including SIDS.

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Abbreviations used in the text

5-HT	5-hydroxytryptamine
SIDS	sudden infant death syndrome

Figure 1.

A-D. Representative 5-HT_{1A} -receptor binding at several levels through the hindbrain illustrate changes in binding with age, and show that the overall pattern of 5-HT_{1A} binding in non-raphe areas in matched control (left columns) and mutant mice (right columns) is similar. Higher magnification images of bracketed regions including those showing raphe nuclei are shown in figure 2. **A.** In P3 control (left) and *Lmx1b*^{ff/p} (right) sections, 5-HT_{1A} binding is generally low (<20 fmol/mg). **B.** At P10, 5-HT_{1A} binding increases are widespread. **C.** By P25, changes in 5-HT_{1A} binding are region-dependent and a more distinctive pattern of binding can be discerned. **D.** Side-by-side sections from matched control (left) and *Pet1*^{-/-} (right) at P25 show a similar pattern to *Lmx1b*^{ff/p} and their controls. **E.** Areas sampled for quantitative analysis indicated by black ovals, IPN= interpeduncular nucleus, DRN= dorsal raphe nucleus, IC=inferior colliculus, CB=cerebellum, RO=raphe obscurus, LPGi= lateral paragigantocellular nucleus, Sp5=spinal trigeminal nucleus. Scale bar in D = 4 mm, all images at same scale.

Figure 2.

High magnification comparison of binding from sections indicated in Figure 1. In controls for *Lmx1b^{f/f/p}*, 5-HT_{1A} binding is associated with the midline within the dorsal raphe nucleus (DRN) at P3 (**A**), and increases at both P10 (**B**) and P25(**C**). In *Lmx1b^{f/f/p}* mutants, loss of midline binding is evident (white arrowheads) at each time-point (**A'**, **B'**, **C'**). In controls 5-HT_{1A} binding again visible in the raphe obscurus (RO) (**D**, **E**, **F**) and nucleus of the solitary tract (**G**). In *Lmx1b^{f/f/p}* mutants, binding is much reduced along the midline at each age in raphe obscurus (**D'**, **E'**, and **F'** white arrowheads). However, 5-HT_{1A} receptor binding persists in the NTS (**G'**, small arrows). At P25, binding in *Pet1*-control dorsal raphe nucleus (**H**) and raphe obscurus (**I**) is similar to *Lmx1b^{f/f/p}* controls. Deficits in midline binding are notable in sections from *Pet1*^{-/-} mice (**H'**, **I'**). All images at the same scale, scale bar = 500 μ m.

Figure 3.

Quantification of 5-HT_{1A}-receptor binding in control and *Lmx1b^{ffp}* mice at each site and age. Significant ($p < 0.05$) differences between consecutive ages are indicated below the X-axis. Y-axis indicates DPAT binding in fmol/mg tissue. Significant differences between genotypes are indicated by asterisk above the relevant measures. In control mice (black lines), 5-HT_{1A}-receptor binding increases between P3 and P10 at every site except the cerebellum. Between P10 and P25, control mice show region-dependent increases or decreases in binding. The dorsal raphe nucleus and raphe obscurus are the only areas with consistent genotype effects.

Figure 4. Quantification of 5-HT_{1A}-receptor binding in control and *Pet1*^{-/-} mice by region and age. Significant (p < 0.05) differences between consecutive ages are indicated below the X-axis. Y-axis indicates DPAT binding in fmol/mg tissue. Significant differences between genotypes are indicated by asterisk above the relevant measures. Between P3 and P8, 5-HT_{1A} receptor binding levels increase at every site in control (black line), but changes are region dependent between P8 and P25. A significant effect of genotype was detected as early as P3 and primarily involved the dorsal raphe nucleus and raphe obscurus.

Figure 5.

Percent retained binding (mutant binding/mean control binding, expressed as percent) in the dorsal raphe and raphe obscurus in both genotypes with age. Each mutant is normalized to its respective control group. At P3 and P8/10, differences between genotypes are not significant. At P25, in both the dorsal raphe and raphe obscurus the loss of 5-HT_{1A} binding in *Lmx1b*^{ff/p} mice was more profound than that seen in *Pet-1*^{-/-} mice. Mean $\pm$ SEM, asterisk indicates $P < 0.05$.