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## Analysis of brain adrenergic receptors in dopamine- $\beta$ -hydroxylase knockout mice

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**Research Report**
**Analysis of brain adrenergic receptors in dopamine- $\beta$ -hydroxylase knockout mice**
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## Abbreviations:

AR, adrenergic receptor

DBH, dopamine- $\beta$ -hydroxylaseDbh, dopamine- $\beta$ -hydroxylase geneGTP $\gamma$ S, 5'-O-(3-[<sup>35</sup>S]thio)

triphosphate

PIC, *para*-iodoclonidine

slm, stratum lacunosum moleculare

of the hippocampus

## ABSTRACT

The biosynthesis of norepinephrine occurs through a multi-enzymatic pathway that includes the enzyme dopamine- $\beta$ -hydroxylase (DBH). Mice with a homozygous deletion of DBH (*Dbh*<sup>-/-</sup>) have a selective and complete absence of norepinephrine. The purpose of this study was to assess the expression of alpha-1, alpha-2 and beta adrenergic receptors ( $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR) in the postnatal absence of norepinephrine by comparing noradrenergic receptors in *Dbh*<sup>-/-</sup> mice with those in *Dbh* heterozygotes (*Dbh*<sup>+/-</sup>), which have normal levels of norepinephrine throughout life. The densities of  $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR were assayed with [<sup>3</sup>H]prazosin, [<sup>3</sup>H]RX21002 and [<sup>125</sup>I]-iodo-pindolol autoradiography, respectively. The  $\alpha_2$ -AR agonist high affinity state was examined with [<sup>125</sup>I]-*para*-iodoclonidine autoradiography and  $\alpha_2$ -AR functionality by  $\alpha_2$ -AR agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S autoradiography. The density of  $\alpha_1$ -AR in *Dbh*<sup>-/-</sup> mice was similar to *Dbh*<sup>+/-</sup> mice in most brain regions, with an up-regulation in the hippocampus. Modest decreases in  $\alpha_2$ -AR were found in septum, hippocampus and amygdala, but these were not reflected in  $\alpha_2$ -AR functionality. The density of  $\beta$ -AR was up-regulated to varying degrees in many brain regions of *Dbh*<sup>-/-</sup> mice compared to the heterozygotes. These findings indicate that regulation of noradrenergic receptors by endogenous norepinephrine depends on receptor type and neuroanatomical region.

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## 1. Introduction

Norepinephrine is a neurotransmitter that signals through alpha-1, alpha-2 and beta adrenergic receptors ( $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR) (Bylund et al., 1994). These are G-protein-coupled receptors whose signaling is important for the regulation of diverse processes within the mature and developing brain (Morris et al., 1983; Lau et al., 1990; Nutt et al., 1997; Troadec et al., 2001). Recently, investigation of noradrenergic regulation of CNS function has been facilitated by the development of mice with a genomic deletion of dopamine- $\beta$ -hydroxylase (DBH), the enzyme necessary for conversion of dopamine to norepinephrine (Thomas et al., 1995). Mice with a homozygous deletion of DBH (*Dbh*<sup>-/-</sup>) have a selective and complete absence of norepinephrine throughout postnatal development and into adulthood, whereas heterozygotes (*Dbh*<sup>+/-</sup>) have normal central norepinephrine levels (Thomas et al., 1995, 1998; Bourdelat-Parks et al., 2005). Prenatally adrenergic agonists as well as the norepinephrine precursor, L-3,4-dihydroxyphenylserine, are provided to pregnant dams via drinking water to assure survival of the *Dbh*<sup>-/-</sup> mice (Thomas et al., 1995).

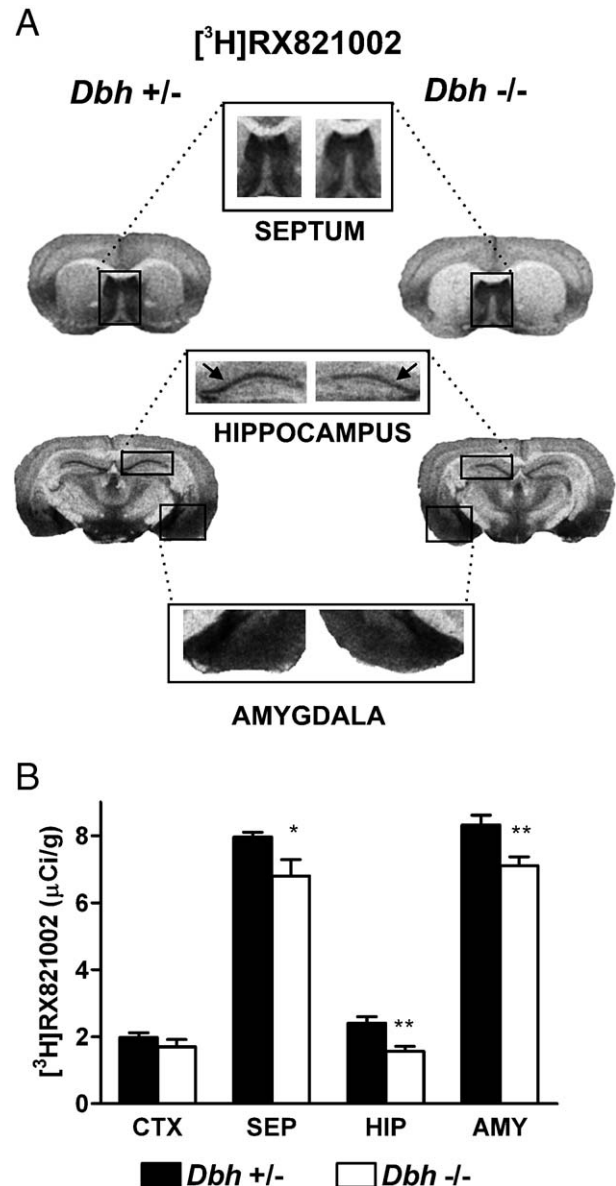
Behaviorally, *Dbh*<sup>-/-</sup> mice manifest a variety of anomalies that point to the importance of norepinephrine in brain function. These include deficits in active-avoidance learning (Thomas and Palmiter, 1997), a failure to respond to antidepressants (Cryan et al., 2004), a sensitization to amphetamine (Weinshenker et al., 2002b) and an increased susceptibility to seizure-inducing stimuli (Szot et al., 1999). Despite the in-depth characterization of behavior in *Dbh*<sup>-/-</sup> mice, little is known about the effects of this deletion on noradrenergic receptor expression, the natural target of norepinephrine. This information will be helpful in interpreting the differing responses of *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice to noradrenergic agents and it will provide data relevant to the role of norepinephrine in regulating the developmental expression of noradrenergic receptors in the postnatal period. In the current studies, we examined differences in the expression of  $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR within the brains of the *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> genotypes, a model of specific and non-traumatic norepinephrine elimination throughout postnatal development and into adulthood (Thomas et al., 1995).

## 2. Results

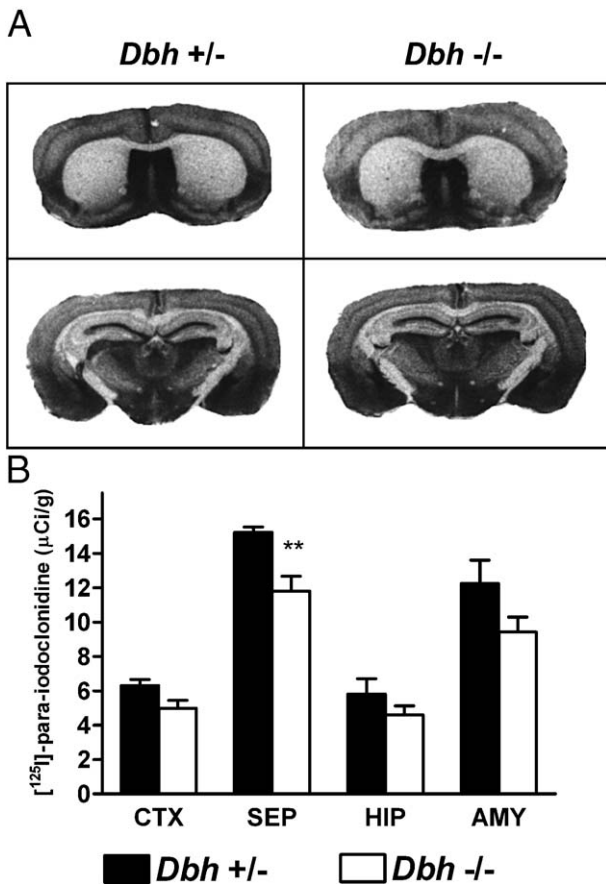
### 2.1. [<sup>3</sup>H]RX821002 autoradiography

The highest densities of  $\alpha_2$ -AR in adult mouse CNS were found in septum and amygdala, with lower densities throughout the cortex and hippocampus, based on [<sup>3</sup>H]RX821002 autoradiography (Fig. 1A). Hippocampal  $\alpha_2$ -AR were most highly expressed in the stratum lacunosum-moleculare (slm) with near undetectable levels in other hippocampal regions (Fig. 1A). In *Dbh*<sup>-/-</sup> mice  $\alpha_2$ -AR were very similar in density to those found in *Dbh*<sup>+/-</sup> mice in the cerebral cortex, based on ligand binding, but there was a small but statistically significant decrease in receptor density in the septum and amygdala (both decreased ~15%; Figs. 1A and B). The greatest difference between heterozygous and homozygous mice was found in

the hippocampus (slm), where there was a 35% decrease in receptor density in *Dbh*<sup>-/-</sup> mice compared to heterozygotes (Figs. 1A and B).



**Fig. 1** –  $\alpha_2$ -Adrenergic receptor densities in CNS regions of *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice. (A) Representative bright field autoradiographs of total [<sup>3</sup>H]RX821002 binding in *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mouse brain. Non-specific binding was 10% or less of total binding and was frequently not detectable. Inserts: higher magnification of [<sup>3</sup>H]RX821002 binding. Decreases in  $\alpha_2$ -AR are found within septum, hippocampus (slm: black arrows) and amygdala of *Dbh*<sup>-/-</sup> mice. (B) Quantification of [<sup>3</sup>H]RX821002 binding within *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mouse brain. *Dbh*<sup>-/-</sup> mice display a significant reduction in [<sup>3</sup>H]RX821002 binding within the septum, hippocampus (slm) and amygdala compared to heterozygotes. CTX=cortex; SEP=septum; HIP=hippocampus; AMY=amygdala. Data are mean  $\pm$  SEM;  $n=4$  for each genotype. \* $p<0.05$ , \*\* $p<0.01$ : two-tailed  $t$  test comparing binding in neuroanatomical regions of *Dbh*<sup>+/-</sup> vs. *Dbh*<sup>-/-</sup> mice.



**Fig. 2** –  $\alpha_2$ -Adrenergic receptor agonist high-affinity state densities in CNS regions of *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice. (A) Representative bright field autoradiographs of total [<sup>125</sup>I]-*p*-iodoclonidine binding in *Dbh*<sup>+/-</sup> and *-/-* mouse brain. Non-specific binding was less than 15% of total binding and was evenly distributed. (B) Quantification of [<sup>125</sup>I]-*p*-iodoclonidine binding within *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mouse brain. There is a significant reduction in [<sup>125</sup>I]-*p*-iodoclonidine binding within the septum of *Dbh*<sup>-/-</sup> mice compared to *Dbh*<sup>+/-</sup> mice. CTX=cortex; SEP=septum; HIP=hippocampus; AMY=amygdala. Data are mean  $\pm$  SEM; *n*=4 for each genotype. \*\**p*<0.01: two-tailed *t* test comparing binding in neuroanatomical regions of *Dbh*<sup>+/-</sup> vs. *Dbh*<sup>-/-</sup> mice.

## 2.2. [<sup>125</sup>I]-*p*-Iodoclonidine autoradiography

We further investigated potential differences in the  $\alpha_2$ -AR by examining the agonist high affinity state of the receptor, the active form of the receptor directly linked to G proteins. For these studies we used [<sup>125</sup>I]PIC, a radioligand that selectively binds to the agonist high affinity state of  $\alpha_2$ -AR when used at very low concentrations, as in these assays. Similar to our findings for total  $\alpha_2$ -AR with [<sup>3</sup>H]RX821002, [<sup>125</sup>I]PIC binding was highest in the amygdala and septum of *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice, with lower levels throughout the cortex and hippocampus (slm) (Figs. 2A and B). The agonist high-affinity state of the  $\alpha_2$ -AR changed very little when norepinephrine was absent. A small, but statistically significant decrease in receptor density was seen only within the septum (~20%; Fig. 2B).

## 2.3. $\alpha_2$ -AR agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S incorporation

Because surprisingly few changes were found in *Dbh*<sup>-/-</sup> animals in total  $\alpha_2$ -AR density or in percentage of receptors in the agonist high affinity state, we examined  $\alpha_2$ -AR agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding to determine if there was a change in receptor linkage to G proteins, the next step in the signal transduction pathway for  $\alpha_2$ -AR. *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice were similar in basal [<sup>35</sup>S]GTP $\gamma$ S binding across brain regions and had a similar level of [<sup>35</sup>S]GTP $\gamma$ S incorporation in response to epinephrine stimulation (Figs. 3A and B). For both *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice, stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding following  $\alpha_2$ -AR stimulation was ~75% above basal for frontal cortex, ~140% for septum and ~30% for hippocampus and amygdala (Figs. 3A and B). The  $\alpha_2$ -AR antagonist, RX821002 (10  $\mu$ M), alone did not affect basal activity in any brain region but did block the ability of epinephrine to stimulate [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 3B), indicating this was an  $\alpha_2$ -AR-specific effect. The similarity in epinephrine-induced [<sup>35</sup>S]GTP $\gamma$ S incorporation between both groups of mice indicates that sensitization of  $\alpha_2$ -AR signal transduction mechanisms, at least at the level of G protein coupling, does not occur in the absence of norepinephrine postnatally.

## 2.4. [<sup>3</sup>H]Prazosin autoradiography

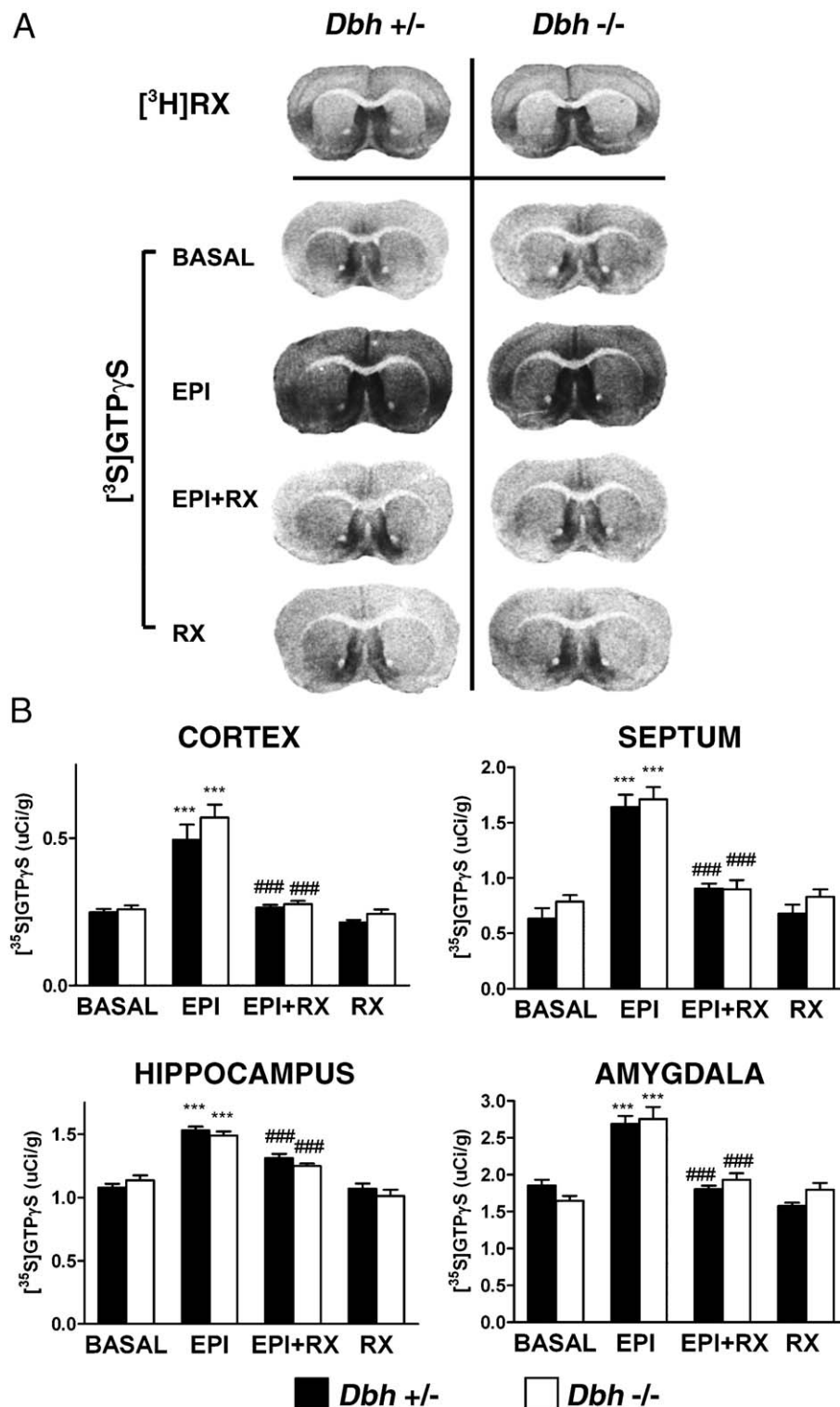
Given the relatively minor changes in  $\alpha_2$ -AR density in animals lacking norepinephrine throughout postnatal development, we examined other adrenergic receptors to determine whether the inability to synthesize norepinephrine would affect their development.  $\alpha_1$ -AR binding sites were found in greatest quantity in the dorsal raphe with moderate levels in the cerebral cortex, bed nucleus of the stria terminalis and thalamus and lowest level in the septum and hippocampus (Figs. 4A and B). [<sup>3</sup>H]Prazosin  $\alpha_1$ -AR binding sites in *Dbh*<sup>-/-</sup> mice did not differ in density from those in *Dbh*<sup>+/-</sup> mice in any region except the hippocampus, where a significant increase (~75%) in binding sites was observed in *Dbh*<sup>-/-</sup> mice (Figs. 4A and B).

## 2.5. [<sup>125</sup>I]Pindolol autoradiography

We also examined  $\beta$ -AR in *Dbh*<sup>-/-</sup> mice. Expression of  $\beta$ -AR was highest in cortex with lower levels in septum and hippocampus. In contrast to the alpha adrenergic receptors,  $\beta$ -AR exhibited a significant up-regulation in all brain regions examined. In the cortex and septum,  $\beta$ -AR were significantly increased ~30% over *Dbh*<sup>+/-</sup> mice, whereas the density of these receptors in the hippocampus was increased by ~50% (Figs. 5A and B).

## 3. Discussion

Many studies have suggested that norepinephrine can play an important role in regulating development of the CNS (Felten et al., 1982; Blue and Parnavelas, 1982; Parnavelas and Blue, 1982). This regulation would be coordinated via norepinephrine's signaling through  $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR (Bylund et al., 1994). Each of these receptors has at least three subtypes and the subtypes possess distinct pharmacological profiles



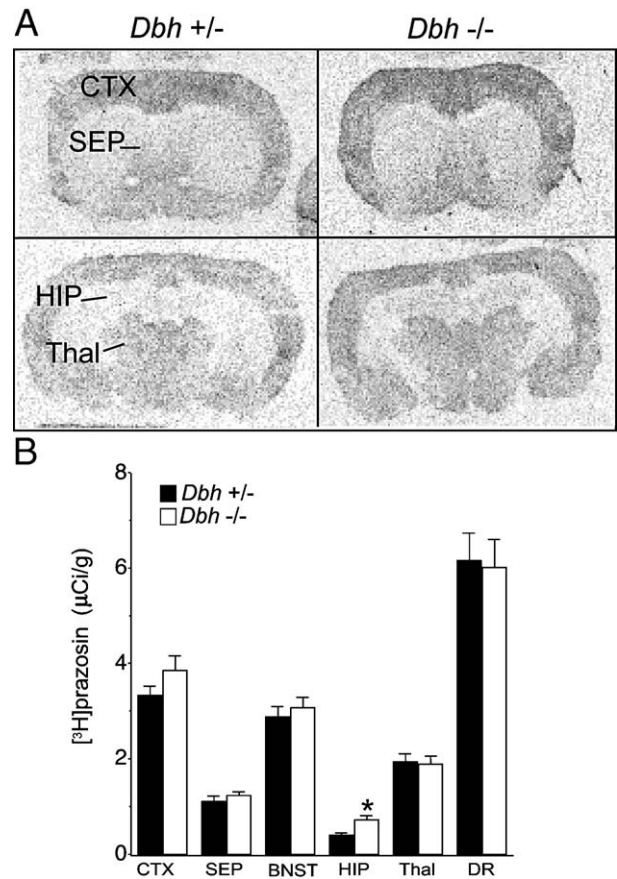
**Fig. 3** -  $\alpha_2$ -Adrenergic receptor-stimulated  $[^3\text{S}]\text{GTP}\gamma\text{S}$  autoradiography in CNS regions of *Dbh +/-* and *Dbh -/-* mice. (A) Representative bright field autoradiographs of total  $[^3\text{H}]\text{RX821002}$  binding (right and left complete sections:  $[^3\text{H}]\text{RX-AR}$ ) and epinephrine-stimulated  $[^3\text{S}]\text{GTP}\gamma\text{S}$  binding within coronal sections (middle inset) of *Dbh +/-* and *Dbh -/-* mice. (B) Quantification of epinephrine-stimulated  $[^3\text{S}]\text{GTP}\gamma\text{S}$  binding in *Dbh +/-* and *Dbh -/-* mice. Despite the developmental absence of norepinephrine in *Dbh -/-* mice, epinephrine-stimulated  $[^3\text{S}]\text{GTP}\gamma\text{S}$  binding is similar between *Dbh -/-* and *Dbh +/-* mice. EPI=epinephrine (100  $\mu\text{M}$ ), RX=RX821002 (10  $\mu\text{M}$ ). Data are mean  $\pm$  SEM;  $n=4$  for each genotype. \*\*\* $p < 0.001$  compared with basal; ### $p < 0.001$  compared to EPI: one-way ANOVA followed by Tukey multiple comparison post hoc test.

and differential distributions within the central nervous system (Bylund et al., 1994) (Zhong and Minneman, 1999, 7388/id; Nicholas et al., 1996, 5798/id; McCune et al., 1993, 5413/id; Rainbow et al., 1984, 6706/id). There are also changes in adrenergic receptor distribution during development, but few have examined receptor subtypes (Jones et al., 1985; Goffinet et al., 1986; Winzer-Serhan and Leslie, 1997; Winzer-Serhan et al., 1997a,b; Happe et al., 2004).

Because adrenergic receptors are the physiological target of norepinephrine, it is reasonable to expect that loss of norepinephrine during postnatal development would lead to changes in these receptors. We examined this possibility using mice lacking DBH, the final enzymatic step in the synthetic pathway for norepinephrine. Although it is necessary for the survival of *Dbh*<sup>-/-</sup> embryos to provide adrenergic receptor stimulation prenatally by providing receptor agonists and DOPS in the drinking water of pregnant dams (see Experimental procedures), these mice completely lack norepinephrine from birth forward (Thomas et al., 1995). In these studies, we compared the *Dbh*<sup>-/-</sup> mice to *Dbh*<sup>+/-</sup> mice, which have been shown to have normal central levels of norepinephrine (Thomas et al., 1995, 1998; Bourdelat-Parks et al., 2005). These animals also have normal localization and density of noradrenergic terminals as indicated by the distribution and density of the norepinephrine transporter (Weinschenker et al., 2002b), a highly specific marker for norepinephrine neurons. This indicates the changes found cannot be ascribed to changes in distribution of noradrenergic terminals.

We found that the postnatal absence of norepinephrine yields differing effects depending on noradrenergic receptor type and brain region. The density of  $\alpha_1$ -AR, indicated by [<sup>3</sup>H]prazosin binding was similar between *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice except for a significant increase in hippocampus in *Dbh*<sup>-/-</sup> mice, in contrast to  $\alpha_2$ -AR. The density changes in  $\alpha_2$ -AR, determined by [<sup>3</sup>H]RX821002 binding, also were relatively minor in the absence of norepinephrine. There were small decreases in  $\alpha_2$ -AR density in septum, hippocampus (slm) and amygdala. However, the agonist high-affinity state of  $\alpha_2$ -AR, examined with [<sup>125</sup>I]PIC binding, was decreased only in septum and the functional linkage of these receptors, measured by agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding, was not significantly altered in any brain region examined. These data suggest that the number of  $\alpha_2$ -AR linked to G proteins remained relatively constant in all brain regions, even though there were small changes in total  $\alpha_2$ -AR density. In contrast to  $\alpha_1$ -AR and  $\alpha_2$ -AR, *Dbh*<sup>-/-</sup> mice respond to the developmental absence of norepinephrine by increasing  $\beta$ -AR expression throughout the brain, with 30% increases in cortex and septum and a 50% increase in hippocampus.

It is important to note that because DBH catalyzes the conversion of dopamine to norepinephrine, *Dbh*<sup>-/-</sup> mice produce and probably release dopamine from their noradrenergic terminals. Therefore, it is possible that dopamine is providing sufficient agonist activity in *Dbh*<sup>-/-</sup> mice to maintain normal  $\alpha_1$ - and  $\alpha_2$ -AR levels. For example, some  $\alpha_2$ -AR subtypes have an affinity for dopamine that is comparable to their affinity for norepinephrine (Zhang et al., 1999). In addition, the “ectopic” dopamine in *Dbh*<sup>-/-</sup> mice appears to help maintain normal expression of the norepinephrine transporter (Weinschenker et al., 2002b). However, a functional



**Fig. 4** –  $\alpha_1$ -Adrenergic receptor densities in CNS regions of *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice. (A) Representative bright field autoradiographs of total [<sup>3</sup>H]prazosin binding in *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mouse brain. Non-specific binding was 10% or less of total binding and was frequently not detectable. (B) Quantification of [<sup>3</sup>H]prazosin binding within *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mouse brain. *Dbh*<sup>-/-</sup> mice display a significant increase in [<sup>3</sup>H]prazosin binding only in the hippocampus when compared to *Dbh*<sup>+/-</sup> mice. BNST=bed nucleus of the stria terminalis; DR=dorsal raphe. Data are mean  $\pm$  SEM; *n*=8 for each genotype. \**p*<0.05: two-tailed *t* test comparing binding in neuroanatomical regions of *Dbh*<sup>+/-</sup> vs. *Dbh*<sup>-/-</sup> mice.

replacement of norepinephrine by dopamine in *Dbh*<sup>-/-</sup> mice is unlikely to explain the maintenance of  $\alpha_2$ -AR levels for three reasons. First, when the selective noradrenergic neurotoxin, *N*-(2-chloroethyl)-*N*-ethyl-2-bromobenzylamine (DSP-4) was used to lesion central noradrenergic neurons in neonatal rats, there was no “ectopic” dopamine and no change in  $\alpha_2$ -AR density (Sanders et al., 2001). Second, *Dbh*<sup>-/-</sup> mice have phenotypes (e.g., seizure susceptibility) that are reversed by  $\alpha_2$  agonists (Szot et al., 2004), which would not be expected if endogenous dopamine could bind to and activate  $\alpha_2$ -AR. Finally, locus coeruleus neurons from *Dbh*<sup>-/-</sup> mice completely lack the characteristic  $\alpha_2$ -AR-mediated autoinhibition observed in locus coeruleus neurons from normal mice, indicating that endogenously released dopamine does not activate  $\alpha_2$ -AR in locus caeruleus of *Dbh*<sup>-/-</sup> mice (C. Paladini

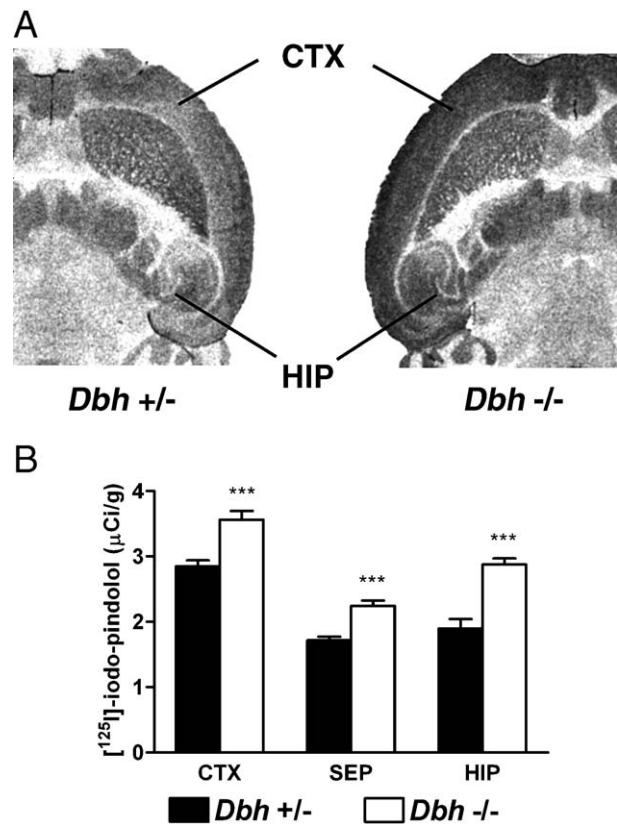
and D. Weinschenker, unpublished observation). The affinity of  $\alpha_1$ -AR and  $\beta$ -AR for dopamine is much lower than for that of norepinephrine, making modulation of these receptors by dopamine highly unlikely (Zhang et al., 2004).

Previous studies have investigated the role of norepinephrine in  $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR ontogeny by lesioning the noradrenergic fibers of neonatal rats with 6-hydroxydopamine (6-OHDA). One study found a ~20% increase in adult  $\alpha_1$ -AR and  $\alpha_2$ -AR in cortical membranes (Dausse et al., 1982) and a separate study showed a dramatic increase in adult  $\beta$ -AR (Lorton et al., 1988). These studies have similarities and differences with our studies. These previous studies found that a neonatal 6-OHDA lesion of noradrenergic neurons lead to a detectable increase in  $\alpha_1$ -AR and  $\alpha_2$ -AR within the cortex of mature rat brain, in contrast to our findings of no change in mouse brain. This may reflect species differences or the effects of the 10–15% of normal norepinephrine remaining following the lesions. Similar to what is found in *Dbh*<sup>-/-</sup> mice, neonatal 6-OHDA caused a persistent up-regulation of  $\beta$ -AR (Lorton et al., 1988). The general pattern of  $\beta$ -AR up-regulation being greater than the effects on  $\alpha_1$ -AR and  $\alpha_2$ -AR is consistent between neonatal 6-OHDA lesion studies in rats and the *Dbh*<sup>-/-</sup> mouse model.

These data from developing brain are in contrast to noradrenergic receptor regulation in the adult brain. In adult animals, a persistent increase in the  $\alpha_2$ -AR high affinity state is elicited as a result of DSP-4 lesion or reserpization, as measured by [<sup>3</sup>H]clonidine binding to membrane preparations (Dooley et al., 1983; Ribas et al., 2001). It is generally found that adult noradrenergic lesions lead to moderate increases in  $\beta$ -AR density (Dooley et al., 1983; Zahniser et al., 1986). In contrast, we find a decrease or no change in total  $\alpha_2$ -AR number, high affinity state or linkage to G proteins following a developmental loss of norepinephrine. We and others have found a persistent increase in  $\beta$ -AR (Lorton et al., 1988) subsequent to a developmental deficiency in norepinephrine, in contrast to the transient increases found in DSP-4 lesioned adults. These findings indicate there are distinct differences in adrenergic receptor regulation in CNS during postnatal development compared to adult brain.

The profile of adrenergic receptor changes in *Dbh*<sup>-/-</sup> mice differs from changes seen in mice with a homozygous deletion of the norepinephrine transporter (*Net*<sup>-/-</sup>) (Gilsbach et al., 2006; Dziedzicka-Wasylewska et al., 2006). Because NET functions to remove released norepinephrine from the synapse, *Net*<sup>-/-</sup> mice display elevated extracellular concentrations of norepinephrine (Vizi et al., 2004). Therefore, *Net*<sup>-/-</sup> mice serve as a model of high noradrenergic tone that differs from the *Dbh*<sup>-/-</sup> mouse model of loss of noradrenergic tone. Adrenergic receptors in *Net*<sup>-/-</sup> mice respond to elevated norepinephrine with decreases in  $\alpha_1$ -AR and  $\beta$ -AR and accompanying increases in  $\alpha_2$ -AR<sub>A</sub> and  $\alpha_2$ -AR<sub>C</sub> subtypes (Gilsbach et al., 2006). These changes are in the opposite direction to the elevated  $\alpha_1$ -AR and  $\beta$ -AR and decreased  $\alpha_2$ -AR we have detected in *Dbh*<sup>-/-</sup> mice. Taken together, these data suggest that  $\alpha_1$ -AR,  $\alpha_2$ -AR and  $\beta$ -AR may respond in an integrated and predictable way to variations in postnatal noradrenergic tone.

The relatively small changes in  $\alpha_1$ -AR and  $\alpha_2$ -AR are consistent with the ability of exogenous adrenergic receptor agonists to modulate neuronal excitability and behavior in *Dbh*<sup>-/-</sup> mice (Sztot



**Fig. 5** –  $\beta$ -Adrenergic receptor densities in CNS regions of *Dbh*<sup>+/+</sup> and *Dbh*<sup>-/-</sup> mice. (A) Representative bright field autoradiographs of total [<sup>125</sup>I]pindolol binding within horizontal sections *Dbh*<sup>+/+</sup> and *Dbh*<sup>-/-</sup> mice. (B) Quantification of [<sup>125</sup>I]pindolol binding within *Dbh*<sup>+/+</sup> and *Dbh*<sup>-/-</sup> mouse brain. In contrast to  $\alpha_1$ -AR and  $\alpha_2$ -AR,  $\beta$ -AR sites are increased in multiple brain regions of *Dbh*<sup>-/-</sup> mice. CTX=cortex, HIP = hippocampus, SEP = septum. Data are mean  $\pm$  SEM; *n*=4 for each genotype. \*\*\**p* < 0.001: two-tailed *t* test comparing binding in neuroanatomical regions of *Dbh*<sup>+/+</sup> vs. *Dbh*<sup>-/-</sup> mice.

et al., 1999; Weinschenker et al., 2001; Murchison et al., 2004). They contrast with the significant changes found in the dopamine system in *Dbh*<sup>-/-</sup> mice. *Dbh*<sup>-/-</sup> mice are hypersensitive to the behavioral effects of D2 agonists and exhibit large increases in the high affinity state of brain D1 and D2 receptors (Weinschenker et al., 2002a; Schank et al., 2005). Our data indicate that  $\alpha_1$ -AR and  $\alpha_2$ -AR are normal in the postnatal absence of norepinephrine and that  $\beta$ -AR expression responds normally to the lack of norepinephrine, whereas other neurotransmitter systems, such as the dopamine system, require norepinephrine for normal development and maintenance.

#### 4. Experimental procedures

[<sup>3</sup>H]RX821002 (58 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). Rauwolscine, isoproterenol, epinephrine bitartrate, RX821002, phentolamine, glycylglycine HCl and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St.



Louis, MO.) [<sup>35</sup>S]GTP $\gamma$ S (1000–1500 Ci/mmol), *p*-[<sup>125</sup>I]iodoclonidine (2200 Ci/mmol), [<sup>125</sup>I]-iodo-pindolol (2200 Ci/mmol) and [<sup>3</sup>H]prazosin (80.5 Ci/mmol) were purchased from Perkin Elmer (Boston, MA). Guanosine 5'-diphosphate sodium (GDP) was purchased from United States Biochemical (Cleveland, OH). All other chemicals were research grade.

#### 4.1. Animals

*Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> mice, maintained on a mixed C57BL6/J and 129SvEv background, were bred as previously described (Thomas et al., 1995). Because *Dbh*<sup>-/-</sup> embryos die in mid-gestation without norepinephrine, adrenergic agonists (phenylephrine and isoproterenol) and the norepinephrine precursor L-3,4-dihydroxyphenylserine (DOPS) were supplied to the embryos via the drinking water of pregnant dams (Thomas et al., 1995). Once *Dbh*<sup>-/-</sup> mice are born, they no longer require pharmacological intervention for viability or development. Thus, the *Dbh*<sup>-/-</sup> mice used in this study lacked norepinephrine from birth and developed postnatally in the complete absence of norepinephrine. Mice were maintained on a 12-h light/dark cycle in a specific pathogen-free facility at the University of Washington (Seattle, WA), and all procedures involving live animals were carried out in this facility. Food and water were available ad libitum. Adult (5–6 months in age) male and female mice were used for all experiments except those involving  $\alpha_1$ -AR, for which all animals were 30 days old. At 30 days of age, these mice are capable of reproduction and their brains are mature by most measurements. Homozygotes and heterozygotes were age-matched and all animals were paired with littermates. Animals were sacrificed by CO<sub>2</sub>, brains rapidly removed and frozen on dry ice. All animal care procedures were in strict accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee. All studies on  $\alpha_1$ -adrenergic receptors were carried out at the University of Washington. All other studies were carried out at the University of Nebraska Medical Center. Each set of experiments was carried out on a separate group of animals.

#### 4.2. [<sup>3</sup>H]RX821002 autoradiography

Autoradiographic studies of  $\alpha_2$ -AR used procedures as previously described (Happe et al., 2004). Briefly, tissue sections 16  $\mu$ m thick were thaw-mounted onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -80 °C. Prior to use, sections were allowed to come to room temperature for 1 h and then were incubated for 1 h at room temperature in 50 mM sodium phosphate buffer, pH 7.4, containing 2.0 nM [<sup>3</sup>H]RX821002. Non-specific binding was determined by the inclusion of 10  $\mu$ M rauwolscine. All incubations except those using [<sup>3</sup>H]prazosin were carried out in plastic slide mailers with 5 slides/9 ml incubation buffer. Slides were washed 2  $\times$  5 min in 50 ml ice-cold buffer, briefly dipped in ice-cold distilled water to remove salts, dried and apposed to tritium sensitive film (Hyperfilm-<sup>3</sup>H, Amersham) for four weeks. Films were developed by standard procedures and resulting images were analyzed with the MCID image analysis system (Imaging Research,

Inc., St. Catharines, Ontario, Canada). Autoradiographic densities were quantified using commercial tritium standards (ART-123; American Radiochemicals, St. Louis, MO), which had been calibrated in this lab to tissue standards (Geary et al., 1985). Neuroanatomy for all studies was assessed by comparing sections to a mouse brain atlas and coronal sections from bregma 0.98 mm to bregma -2.54 mm were analyzed (Franklin and Paxinos, 1997). All data for each region represent the mean of at least two determinations from separate sections for each of three to four animals.

#### 4.3. [<sup>125</sup>I]-*para*-iodoclonidine autoradiography

Sections from the same regions as those described for [<sup>3</sup>H]RX821002 autoradiography were used. Sections were incubated in 50 mM Tris-HCl, 120 mM sucrose, pH 7.4, with 50 pM [<sup>125</sup>I]*para*-iodoclonidine (PIC) as ligand. Non-specific binding was determined by addition of 10  $\mu$ M rauwolscine. Sections were incubated for 3 h at room temperature in a horizontal slide mailer with 5 slides/9 ml incubation buffer. For studies with [<sup>125</sup>I]PIC, mailers were placed on a shaking water bath platform, and every 15 min the mailers were gently inverted three times to ensure homogenous distribution of the radioligand. After incubation sections were washed two times for 5 min in ice-cold 50 mM Tris-HCl, pH 7.4, briefly dipped in ice-cold distilled water and rapidly dried under a stream of cool air. Sections were apposed to Biomax MR film (Eastman Kodak Co., Rochester, NY) for 48 h. Films were developed by standard procedures and analyzed using the MCID system, the mouse brain atlas and commercial tritium standards (American Radiochemicals, St. Louis, MO) that were individually calibrated to <sup>125</sup>I-tissue standards (Miller and Zahniser, 1987).

#### 4.4. $\alpha_2$ -AR agonist-stimulated GTP $\gamma$ S autoradiography

Linkage of  $\alpha_2$ -AR to G proteins was measured using the agonist-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding assay (Sim et al., 1995), as optimized for  $\alpha_2$ -AR (Happe et al., 2001). Slide mounted 16  $\mu$ m tissue sections from the same regions as those described for [<sup>3</sup>H]RX821002 autoradiography were hydrated in assay buffer (50 mM glycylglycine, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, 100 mM NaCl, pH 7.5) at room temperature for 10 min. This was followed by incubation in assay buffer plus 2 mM GDP for 30 min.  $\alpha_2$ -AR were stimulated by incubation with 100  $\mu$ M epinephrine for 2 h in assay buffer with 0.1 nM [<sup>35</sup>S]GTP $\gamma$ S, 2 mM GDP and 0.2 mM DTT. Addition of 10  $\mu$ M RX821002, a specific  $\alpha_2$ -AR antagonist with similar affinity for all three receptor subtypes, was used to determine non-specific binding of [<sup>35</sup>S]GTP $\gamma$ S. Sections were washed 2  $\times$  5 min in ice-cold 50 mM glycylglycine, 0.2 mM DTT, pH 7.5; rinsed briefly in ice-cold distilled water; dried under a stream of cool air; and apposed to  $\beta$ max Hyperfilm (Amersham) for three days. Films were developed by standard procedures and analyzed using the MCID system, the mouse brain atlas and commercial tritium standards (American Radiochemicals, St. Louis, MO) that were individually calibrated in this lab to [<sup>35</sup>S]-tissue standards (Miller and Zahniser, 1987). All data for each region represent the mean of at least two determinations for each of three to four animals.

#### 4.5. [<sup>3</sup>H]Prazosin autoradiography

Autoradiographic studies of  $\alpha_1$ -AR used procedures as previously described (Szot et al., 2005). Briefly, tissue sections 16  $\mu$ m thick were thaw-mounted onto Superfrost Plus slides and stored at  $-80^\circ\text{C}$ . Slides were thawed at room temperature for 10 min and then 400  $\mu$ l/slide of incubation buffer ( $\sim 0.2$  nM [<sup>3</sup>H]prazosin in 50 mM Tris buffer, 1 mM EDTA, pH 7.4) was placed over the tissue. Non-specific binding was defined in the presence of 10  $\mu$ M phentolamine. Slides were incubated for 40 min at room temperature, washed twice for 2 min in ice-cold 50 mM Tris buffer, pH 7.4, dipped in ice-cold distilled water to remove salts and then rapidly dried under a stream of cool air. Slides were apposed to Biomax MR film (Eastman Kodak Co.) for 8 weeks. Films were developed by standard procedures and the resulting images were analyzed using the MCID system, the mouse brain atlas and commercial tritium standards. All data for each region represent the mean of at least three determinations for each of the eight *Dbh*<sup>-/-</sup> and *Dbh*<sup>+/-</sup> animals.

#### 4.6. [<sup>125</sup>I]-Iodo-pindolol autoradiography

Autoradiographic studies of  $\beta$ -AR were carried out by incubating 16  $\mu$ m slide-mounted horizontal tissue sections in 50 mM Tris, pH 7.4 with 50 pM [<sup>125</sup>I]-iodo-pindolol for 1 h at room temperature. Non-specific binding was defined by inclusion of 100  $\mu$ M isoproterenol. Following incubation, slides were washed 3  $\times$  5 min in ice-cold buffer and a quick rinse in ice-cold distilled water. Sections were dried under a stream of cool air and apposed to BioMax film (Kodak) for 24 h. Films were developed by standard procedures and analyzed using the MCID system, the mouse brain atlas and commercial tritium standards (American Radiochemicals, St. Louis, MO) that were individually calibrated to [<sup>125</sup>I]-tissue standards. All data for each region represent the mean of at least two determinations for each of three to four animals.

#### 4.7. Statistics

Data from receptor autoradiography experiments comparing *Dbh*<sup>+/-</sup> and *Dbh*<sup>-/-</sup> mice were analyzed with Student's two-tailed t test. Epinephrine-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding data were analyzed by one-way ANOVA with the Tukey multiple comparison post hoc test.

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