

Pharmacologic and Genetic Characterization of Two Selective Serotonin
Transporter Ligands: 2-[2-(dimethylaminomethylphenylthio)]-5-
fluoromethylphenylamine (AFM) and 3-amino-4-[2-
(dimethylaminomethylphenylthio)]benzotrile (DASB)

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Running Title: Selectivity and pharmacological profile of AFM and DASB

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Abbreviations:

AFM: 2-[2-(dimethylaminomethylphenylthio)]-5-fluoromethylphenylamine

DASB: 3-amino-4-[2-(dimethylaminomethylphenylthio)]benzonitrile

SERT: Serotonin transporter

SRIs: Serotonin reuptake inhibitors

PET: Positron emission tomography

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Abstract

The expression and function of the serotonin transporter (SERT) is important in the regulation of mood and emotion. Determination of SERT alterations in physiological and pathological states is essential for understanding the role of SERT in mood regulation, and in the etiology and therapy of psychiatric disorders. Two SERT ligands, AFM and DASB, have recently been developed for PET imaging. The aim of the present study was to determine the selectivity of these compounds for SERT. Autoradiography of ^3H -AFM or ^3H -DASB binding was compared in the brains of mice with genetically normal, diminished or absent 5-HT transporters. In addition, the pharmacodynamic profile of ^3H -AFM was examined in the mouse brain. The distribution of ^3H -AFM and ^3H -DASB binding in the normal brains was consistent with that of previously studied serotonin reuptake inhibitors (SRIs). Both ligands had negligible binding in the brain of SERT knockout mice and binding was reduced approximately 50% in heterozygote SERT mice. The K_d of ^3H -AFM binding in the cortex and midbrain was 1.6 and 1.0 nM, respectively. Competition studies showed that ^3H -AFM has very low affinity for norepinephrine and dopamine transporters as well as 5-HT receptors, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors. In addition, fenfluramine showed a low capability to compete with ^3H -AFM. The present results suggest that both AFM and DASB are highly selective SERT ligands potentially suitable for use in human PET studies of SERT.

Introduction

The function of the serotonin transporter (SERT) is to reuptake 5-HT from synaptic cleft back into serotonin nerve terminals for enzymatic degradation or recycling. By doing so, SERT controls the concentration of 5-HT in the synaptic cleft and perisynaptic regions and thus its actions at 5-HT receptors. Studies have shown that the function of SERT is important in regulation of emotional states. For example, a polymorphism in the promoter region of human SERT (5HTTLPR) that alters the expression of SERT may be related to personality traits and to some psychiatric disorders including depression (Lesch et al., 1996; Murphy et al., 2001; Caspi et al., 2003). In addition, SERT is the target of serotonin reuptake inhibitors (SRIs), a group of widely used antidepressants (Owens, 1997). Therefore, *in vivo* studies of SERT may help us to understand its role in normal physiology and in pathological states.

Positron emission tomography (PET) allows the *in vivo* measurement of the density and distribution of a target protein (Mountz et al., 2002; Parsey and Mann, 2003). Clear interpretation of PET imaging results depends, therefore, on the selectivity of the probe for its target protein. AFM (2-[2-(dimethylaminomethylphenylthio)]-5-fluoromethylphenylamine) and DASB (3-amino-4-[2-(dimethylaminomethylphenylthio)]benzonitrile) are new compounds recently developed as SERT ligands (Ginovart et al., 2003; Houle et al., 2000; Huang et al., 2002b). Although they have been used in PET scans of humans and monkeys, relatively little information has been reported on their pharmacological profile. Although *in vitro* pharmacological studies of DASB have been reported (Wilson et al., 2002), no data are available for the pharmacological profile of AFM. In addition, Wilson et al showed that fluoxetine (up to 1 mg/kg) did not completely block ¹¹C-DASB binding in rats (Wilson et al., 2002). A likely reason for this incomplete blockade is that the dose of fluoxetine was not high enough to block all SERT

binding sites, since complete blockade of SERT sites requires at least 10 mg/kg fluoxetine in rats (Fuller et al., 1978). However, it is necessary to evaluate the specificity of these two ligands. In the present studies, we used SERT knockout mice as a “clean” pharmacological state to examine the selectivity of these two PET ligands for SERT. Autoradiography was performed with ^3H -AFM and ^3H -DASB using brain sections from wildtype and SERT mutant mice. In addition, we examined the pharmacological parameters of AFM using ^3H -AFM homogenate binding.

Methods

Animals

Female SERT mutant mice from a 129sv/ev background (Bengel et al., 1998) were used for the autoradiographic studies. The SERT mutant mice and their SERT normal littermates were from the F9 generation and were five to eight months of age, with body weights of 25-35 g. In the homogenate binding studies, normal male C57/B6 mice were used. The mice were housed in groups of four to five per cage in a light- (12 hr light/dark, lights on at 6 a.m.), humidity- and temperature-controlled room. Food and water were available *ad lib*. All animal procedures were approved by the NIMH Animal Care and Use Committee.

Materials

³H-AFM and ³H-DASB were custom labeled by American Radiochemicals, Inc. (St. Louis, MO). Demethyl precursors of AFM and DASB were synthesized by Henry Huang (Columbia University) and Alan Wilson (University of Toronto)(Wilson et al., 2000), respectively. Citalopram, nisoxetine, 8-OH-DPAT, ketanserin, mianserin and fenfluramine were purchased from Sigma-RBI (St. Louis, MO). RU 24969 and RS 102221 were purchased from Tocris (Ballwin, MO.).

Autoradiographic studies

Preparation of brain sections

Female mice with intact (+/+), heterozygous (+/-) and homozygous knockout (-/-) SERT genes were decapitated. The whole brains were removed and frozen immediately in dry ice-cooled isopentyl alcohol for 10 seconds. The brains were then placed on dry ice for 10 min until they were completely frozen. Brains were wrapped and stored at -80° C until sectioning.

The mouse brain was cut into 16 μm -thick coronal sections in a cryostat. The sections were thaw-mounted onto chromalum/gelatin-coated glass slides and stored at -80°C for studies within one month. To limit variation among the slides, each slide contained brain sections from 3 mice (one genotype each). Three levels of sections were collected: hippocampus (bregma -1.34 to -1.94 mm), midbrain (bregma -4.36 to -4.84 mm) and cerebellum (bregma -5.20 to 5.40 mm) according to a mouse brain atlas (Franklin and Paxinos, 1997).

Autoradiography for ^3H -AFM and ^3H -DASB binding:

Brain sections were preincubated for 30 min at room temperature in assay buffer (50 mM Tris (pH 7.4) containing 150 mM NaCl and 5 mM KCl). Slides were then incubated for 2 hours at room temperature with ^3H -AFM or ^3H -DASB (1.0 nM). Non-specific binding was defined in the presence of 10^{-5}M paroxetine. The slides were then washed twice with assay buffer at 4°C for 10 min and rinsed with cold double-distilled H_2O . After air blow-drying, the slides were exposed to ^3H -Hyperfilm (Amersham, IL) for two months. A set of ^3H microscales (Amersham, IL) was exposed with the slides to calibrate the optical density readings into fmol/mg tissue equivalent. The density of ^3H -AFM or ^3H -DASB binding sites in the brain regions was analyzed using the MCID image analysis program (Imaging Research Inc, Ontario, Canada).

Homogenate binding of ^3H -AFM

The homogenate binding assay of ^3H -AFM was modified from Wilson et al. and Li et al. (Wilson et al., 2000; Li et al., 1997). Whole cortex or midbrain were dissected immediately after decapitation of normal mice and were stored in -80°C until use. The cortex or midbrain were homogenized in 10 mL assay buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl and 5 mM KCl) with Tissue Tearor homogenizer at top speed for about 10 s at 4°C . The homogenates were centrifuged at 35,000xg for 20 min at 4°C . The pellets were resuspended to 10 mL assay

buffer and centrifuged at 35,000xg for 20 min. The final pellet was resuspended in assay buffer (30 mg tissue/mL). Protein concentrations of the homogenates were determined by the Lowry assay (Lowry et al., 1951).

For saturation studies, 50 μ l tissue homogenate were incubated with ^3H -AFM in total volume of 1 mL assay buffer for 2 hours at room temperature. Six concentrations of ^3H -AFM (between 0.5-15 nM for midbrain and 0.5-8nM for cortex) were used in the assay. Non-specific binding was defined in the presence of 10^{-5} M paroxetine (which was about 50% of total binding using 0.5 nM ligands). The incubated solution was then filtered with Whatman GF/C filter paper (pre-treated with 0.5 mM Tris-HCl, pH 7.4) and washed three times with 4 ml cold 0.5 mM Tris-HCl, pH 7.4. The filter paper was counted in 5 mL scintillation fluid (Ecolife, ICN). The dissociation constant K_d and receptor density B_{max} were calculated by non-linear regression using the Prism (version 4) program (GraphPad Software Inc. San Diego, CA).

For competition studies, 50 μ l cortex homogenate were incubated with 2 nM ^3H -AFM in total volume of 1 ml assay buffer containing 10^{-12} - 10^{-7} M 5-HT transporter inhibitors (paroxetine and citalopram) or 10^{-10} - 10^{-4} M other drugs. Nonspecific binding was defined in the presence of 10^{-5} M paroxetine except that 10^{-5} M citalopram was used in the competition study for paroxetine. Solutions were incubated for 2 hours at room temperature and filtered as described above. K_i values and Hill slopes were calculated by one site competition nonlinear regression using the Prism program. In the case when the Hill slope was less than 1, two site competition nonlinear regression was used to further evaluate the possibility of two binding sites.

Results

Autoradiography of ³H-AFM and ³H-DASB

High densities of ³H-AFM and ³H-DASB binding were observed in the lacunosum molecular layer of hippocampic CA1 region, thalamus, raphe nuclei and locus ceruleus of SERT +/+ mice (Fig 1, Fig 2 and Table 1). The binding sites were reduced about 50% in SERT +/- mice (Table 1). There was no detectable specific binding site for either ³H-AFM or ³H-DASB in any of the brain sections of SERT -/- mice (Fig.1 ,Fig.2 and Table 1), i.e. the specific binding sites was essentially zero in all regions (Table 1). As expected, in SERT +/+ mice, both ligands showed a relatively high binding density in the substantia nigra (data not shown) and locus ceruleus (Fig.1, Fig.2 and Table 1). However, ³H-AFM and ³H-DASB had little binding in these two regions of SERT -/- mice (Fig.1, Fig.2 and Table 1).

Pharmacological studies for ³H-AFM

The Kd and Bmax of ³H-AFM in the whole cortex and midbrain were examined using a saturation assay. Saturation curves and Scatchard plots are presented in Fig. 3. The Kd of ³H-AFM in the cortex [1.6 ± 0.09 nM (n=4)] was significantly higher than that in the midbrain [1.0 ± 0.17 nM (n=4)]. The Bmax of ³H-AFM in the cortex and midbrain was 454.6 ± 29.0 and 175.7 ± 28.6 fmol/mg protein, respectively. Hill slopes for the ³H-AFM binding in the cortex and midbrain were 1.04 and 0.95, respectively.

As shown in the Table 2 and Fig 4, ³H-AFM can be displaced by two 5-HT transporter inhibitors, paroxetine and citalopram, with much higher affinities than those for the dopamine transporter inhibitor, GBR 12935, and for norepinephrine transporter inhibitors, desipramine and nisoxetine. However, Ki values for paroxetine and citalopram in ³H-AFM binding were 10 times higher than their Kds. Also, the Hill slope for paroxetine and citalopram were about 0.8 (Table

2). Analyzing the data with competition nonlinear regression for two binding sites showed two K_i values for citalopram (5.7 nM and 231nM, respectively), but one K_i for paroxetine. The 5-HT_{2A} or 5-HT_{2C} antagonists, ketanserin and RS 100221 had very low affinity for ³H-AFM binding sites (Table 2 and Fig 5). Furthermore, the 5-HT_{1A} and 5-HT_{1B} agonists, 8-OH-DPAT and RU 24969, showed low affinity for ³H-AFM binding sites. Fenfluramine, a 5-HT releaser, also displaced ³H-AFM with low affinity (pK_i = 5.2).

Discussion

The present results demonstrate that AFM and DASB are selective SERT ligands. Their binding can be displaced by selective serotonin reuptake inhibitors but not by drugs selective for the other two major monoamine transporters (dopamine and norepinephrine). The K_d of AFM in the midbrain observed in the present study was consistent with its K_i value determined by displacement of AFM binding to ^3H -paroxetine sites (Huang et al., 2002a). Furthermore, AFM was found to have very low affinity for 5-HT receptors, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors.

In the present study, we used SERT knockout mice as a tool to study the selectivity of AFM and DASB. SERT knockout mice were created by disrupting the second exon of the 5-HT transporter gene. Thus, SERT knockout mice do not express intact 5-HT transporters and cannot bind SERT ligands including RTI-55 and citalopram (Bengel et al., 1998). Our hypothesis was that if AFM and DASB selectively bind to SERT, their specific binding to the brain of SERT knockout mice should be undetectable. Our results showed that there is no significant binding of ^3H -AFM and ^3H -DASB in the brain sections of SERT knockout mice compared to their normal littermates (Fig. 2, Fig 3 and Table 1). The concentration of 1 nM ^3H -AFM and ^3H -DASB used in the present study is at least ten times higher than that achieved in a typical PET scan, suggesting that the *in vivo* binding sites of these ligands are predominantly SERT.

SERT binds several types of antidepressants, including SRIs, such as paroxetine and citalopram and some of tricyclic antidepressants (TCAs), such as imipramine and clomipramine. Among these ligands, SRIs have higher affinity and greater selectivity than TCAs (Lane and Baker, 1999; Levy and Van de Kar, 1992). ^3H -imipramine has both high and low affinity binding sites, while ^3H -paroxetine has only one binding site in rat brain (Hrdina et al., 1990; Hrdina,

1987). The autoradiographic distribution of ^3H -AFM and ^3H -DASB binding was more similar to ^3H -paroxetine than to ^3H -imipramine binding (Hrdina et al., 1990). In addition, competition studies showed that both citalopram and paroxetine have a high potency to compete with ^3H -AFM (Table 2 and Fig.4). The present data were also consistent with others indicating that the affinity of paroxetine is about ten-time higher than that of citalopram (Wong et al., 1995). These results suggest that AFM and DASB are highly selective SERT ligands.

Several monoamine transporter inhibitors have high affinity for SERT, the dopamine transporter (DAT) and/or the norepinephrine transporter (NET). For example, cocaine and several its analogs, such as RTI-55, have high affinity for SERT and dopamine transporters (Silverthorn et al., 1994;Shearman et al., 1996;Boja et al., 1992). Duloxetine has high affinity for both SERT and NET (Bymaster et al., 2001;Fuller et al., 1994). Therefore, it is important to evaluate the affinity of new ligands for DAT and NET. We expected if these compounds have high affinity for DAT and NET, we should detect binding sites in substantia nigra (DAT) and locus ceruleus (NET) of SERT $-/-$ mice, which have intact DAT and NET. As shown in Fig 1 and Fig 2, there are no detectable binding sites of ^3H -AFM and ^3H -DASB in the locus ceruleus of SERT $-/-$ mice. Our preliminary study (data not shown) also showed that ^3H -AFM and ^3H -DASB binding was not detectable in the substantia nigra of SERT $-/-$ mice. Furthermore, competition studies demonstrated that the dopamine reuptake inhibitor GBR 12935 and the norepinephrine reuptake inhibitors desipramine and nisoxetine have very low affinity for ^3H -AFM binding sites (Table 1 and Fig 3). These results suggest that AFM and DASB bind selectively to 5-HT transporters, but not to dopamine or norepinephrine transporters.

Some SRIs, such as fluoxetine, have relative high affinity for 5-HT_{2C} and/or 5-HT_{2A} receptors (Palvimaki et al., 1996;Wong et al., 1995). We examined the affinities of AFM for 5-

HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors. The K_i values of all the agonists and antagonists tested in the present studies are at least 100 times higher than those for SRIs (Table 2 and Fig. 5). Unlike fluoxetine, AFM has almost undetectable binding to 5-HT_{2A} receptors (K_i for ketanserin is undetectable) and very low affinity for 5-HT_{2C} receptors (K_i of RS 102221 is 1.7 x 10⁻⁵ M).

The present results showed that the Hill slopes for citalopram and paroxetine competition curve were less than one. In addition, the K_d value for ³H-AFM binding in the cortex was higher than that in the midbrain. Furthermore, the ³H-AFM binding sites in the cortex were much lower than that in the dorsal raphe in the autoradiographic assay (1 nM AFM), while in the homogenate binding studies, the B_{max} of ³H-AFM binding in the cortex was much higher than that in the midbrain. Although the higher B_{max} of ³H-AFM binding in the cortex than that in the midbrain determined by homogenate assay could be due to that high SERT density in the raphe nuclei are diluted by their surround tissue that has low SERT, the different between the two regions is too high to be explained by the dilution effect. Thus, these data suggest that ³H-AFM might have two binding sites in the cortex that can be displaced by the SERT inhibitors, paroxetine and citalopram. The K_i values for these binding sites are in the nanomolar range, which are much smaller than that for DAT and NET inhibitors, as well as for agonists and antagonists of 5-HT receptors measured in the present studies. On the other hand, paroxetine and citalopram are highly selective SERT inhibitors. Besides the serotonin transporter, no other transporters or receptors have an affinity for citalopram in nanomolar range. Furthermore, there is no detectable binding site for ³H-AFM in SERT knockout mice. Together, it is unlikely that the two binding sites of ³H-AFM are due to AFM binding to other transporter or receptors. One possible explanation is that ³H-AFM binds to two sites on SERT. However, further studies are required to evaluate this possibility.

Fenfluramine is an amphetamine derivative and functions as a 5-HT releaser. Its action requires 5-HT transporters and can be blocked by SRIs (Baumann et al., 1998; Rothman and Baumann, 2002). Two mechanisms may be involved in fenfluramine-induced 5-HT release. At low concentrations, fenfluramine stimulates a Ca^{+2} -dependent 5-HT release, suggesting that this 5-HT release is mediated by an exocytotic process (Cinquanta et al., 1997a). At high concentrations, fenfluramine-induced 5-HT release is mediated by a Ca^{+2} -independent process in which fenfluramine may exchange with 5-HT through SERT (Cinquanta et al., 1997b; Rudnick and Wall, 1993; Levi and Raiteri, 1993). However, it is unclear whether the action of fenfluramine requires binding to SERT at SRI binding sites. We performed a competition study using fenfluramine to displace ^3H -AFM binding. Our results showed that fenfluramine has little capacity to bind to 5-HT transporter, at least at the site labeled with ^3H -AFM. It was reported that amphetamine derivatives, such as p-chloroamphetamine and 3,4-methylenedioxymethamphetamine (MDMA), bind to SERT with an affinity about 10^{-7}M . (Battaglia et al., 1988; Rudnick and Wall, 1992). It is interesting that fenfluramine has a low affinity for SERT. However, we can not rule out that fenfluramine binds to SERT through other sites rather than AFM binding sites. Further studies are required to clarify this possibility.

In the present studies, we demonstrated that AFM and DASB selectively bind to SERT. AFM has very low affinity for norepinephrine transporters and dopamine transporters. The affinities of AFM for 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A} and 5-HT_{2C} receptors are much lower than those of SRIs. It will be interesting to know what are the pharmacologic effects of these ligands. For example, are they SERT inhibitors or enhancers? Do they have the potential to be used as antidepressants? Future studies should be pursued to address these questions.

In conclusion, the present results demonstrate that AFM and DASB are selective ligands for SERT in mouse brain and offer supportive data validating their use as PET ligands for SERT in humans and other species. Furthermore, several questions for future studies are raised from the present results, which will help us to understand the pharmacological properties of these ligands.

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Figure Legends:

Figure 1. Autoradiography of ^3H -AFM binding to coronal brain sections of SERT mutant mice. Left panel: wild-type SERT mice (+/+); middle panel: heterozygous SERT knockout mice (+/-); right panel: homozygous SERT knockout mice (-/-). ^3H -AFM (1 nM) was used in the assay (Total). Non-specific binding (NSB) was defined in the presence of 10^{-5} M paroxetine. The nonspecific binding sites were about 5-50% of total binding in various brain regions. **CA1**: CA1 region of hippocampus; **CL**: locus ceruleus. CTX: cortex; **DR (MB)**: dorsal raphe in the midbrain section; **DR (cer)**: dorsal raphe in the cerebellum section; **LPT**: lateral posterior thalamus; **MR**: median raphe; **PH**: posterior hypothalamic area and **PV**: paraventricular thalamus.

Figure 2. Autoradiography of ^3H -DASB binding to coronal brain sections of SERT knockout mice. Left panel: wild-type SERT mice (+/+); middle panel: heterozygous SERT knockout mice (+/-); right panel: homozygous SERT knockout mice (-/-). ^3H -DASB (1 nM) was used in the assay (Total). Non-specific binding (NSB) was defined in the presence of 10^{-5} M paroxetine. The nonspecific binding sites were about 10-50% of total binding in various brain regions

Figure 3. Saturation study of ^3H -AFM binding in the midbrain and cortex. Insertions are Scatchard plots for the two saturation curves. The data were presented as mean of four mice.

Figure 4. Competition curves of monoamine transporter inhibitors to ^3H -AFM binding in the cortex of normal mice. The cortex membranes were incubated with 2 nM ^3H -AFM in the

presence of 10^{-12} - 10^{-7} M 5-HT transporter inhibitors or 10^{-10} - 10^{-4} M norepinephrine and dopamine transporter inhibitors. Each curve represents the mean of 3-4 mice.

Figure 5. Competition curves of 5-HT agonists and antagonists to ^3H -AFM binding in the cortex of normal mice. The cortex membranes were incubated with 2 nM ^3H -AFM in the presence of 10^{-10} - 10^{-4} M of 5-HT agonists and antagonists. Each curve represents the mean of 2-4 mice.

Table 1 Autoradiography of ³H-AFM and ³H-DASB binding in the brain of SERT mutant mice

Brain Region	³ H-AFM binding (nCi/mg tissue)		
	SERT +/+	SERT +/-	SERT -/-
CA1	0.92	0.62	0.04
PH	2.07	0.98	-0.02
LPT _h	1.13	0.73	-0.02
PV	1.40	0.72	-0.14
CTX	0.42	0.32	0.05
DR (MB)	3.45	1.29	0.00
MR	1.48	0.71	0.00
CL	2.32	1.39	0.04
DR (cer)	3.19	1.70	-0.05

Brain Region	³ H-DASB binding		
	SERT +/+	SERT +/-	SERT -/-
CA1	0.83	0.41	0.14
PH	1.61	0.89	0.09
LPT _h	0.97	0.53	-0.06
PV	1.50	0.62	0.13
CTX	0.56	0.21	0.01
DR (MB)	3.54	1.92	0.08
MR	1.78	0.62	-0.02
CL	0.41	0.24	0.08
DR (cer)	1.10	0.64	-0.11

The data are presented as means of three brain sections.

Abbreviations are same as listed in Fig 1

Table 2. Competition studies of ³H-AFM binding in mouse cortex membranes

Compound	K _i (nM)	Hill slope	Selectivity
Paroxetine	4.7 ± 0.9	0.77	5-HT reuptake inhibitor
Citalopram	19.5 ± 6.4	0.80	5-HT reuptake inhibitor
Nisoxetine	7046 ± 1290	1.1	Norepinephrine reuptake inhibitor
Desipramine	8114 ± 1548	1.07	Norepinephrine reuptake inhibitor
GBR 12935	25540 ± 1220	1.1	Dopamine reuptake inhibitor
8-OH-DPAT	31145 ± 8715	1.09	5-HT _{1A} agonist
RU 24969	3006 ± 573	0.9	5-HT _{1A/1B} agonist
Ketanserin	Nd*	Nd	5-HT _{2A} antagonist
RS 102221	17030 ± 3000	Nd	5-HT _{2C} antagonist
Mianserin	77740 ± 26160	1.4	5-HT ₂ antagonist
Fenfluramine	7047 ± 1905	0.9	5-HT releaser

The data were obtained from non-linear regression analysis for one binding site. The data are presented as mean ± SEM (n=3-4).

* Nd: not detectable.

^3H -AFM Binding

SERT +/+

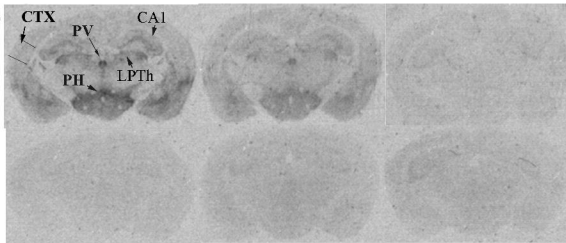
+/-

-/-

Hippocampus

Total

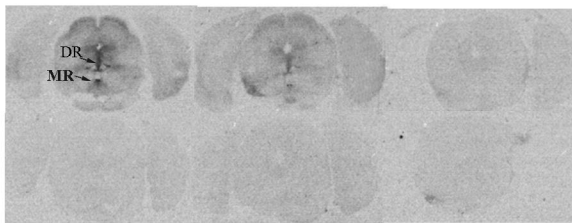
NSB



Midbrain

Total

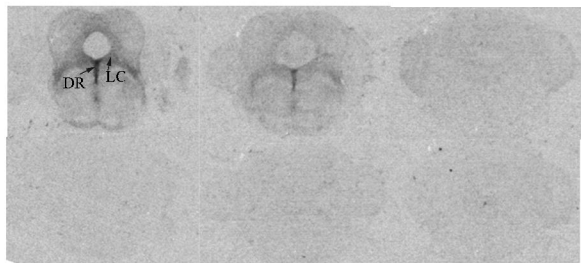
NSB



Cerebellum

Total

NSB



DASB 1.0 nM

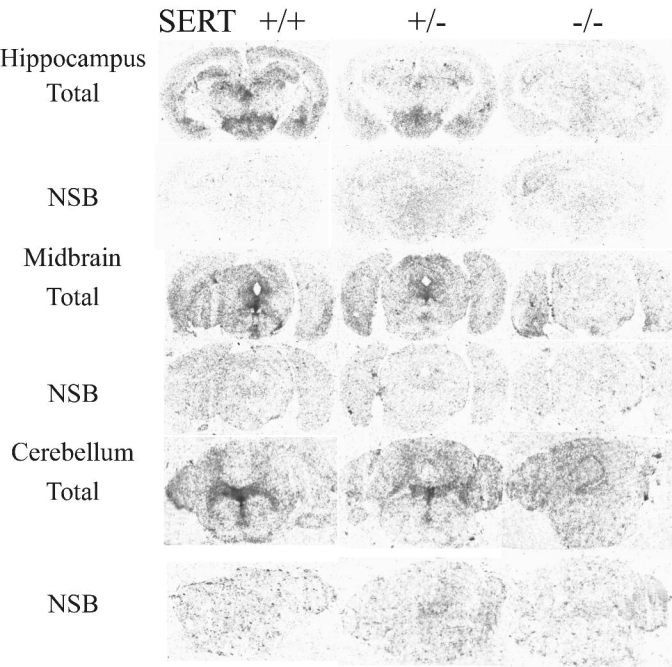
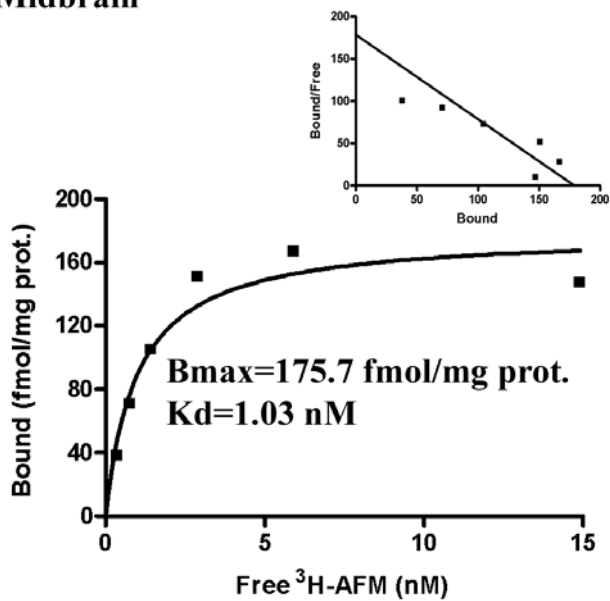


Figure 3

Midbrain



Cortex

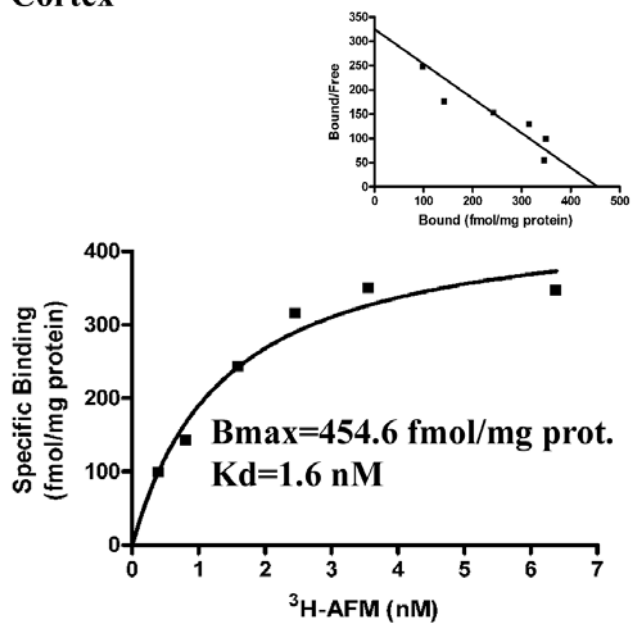


Figure 4

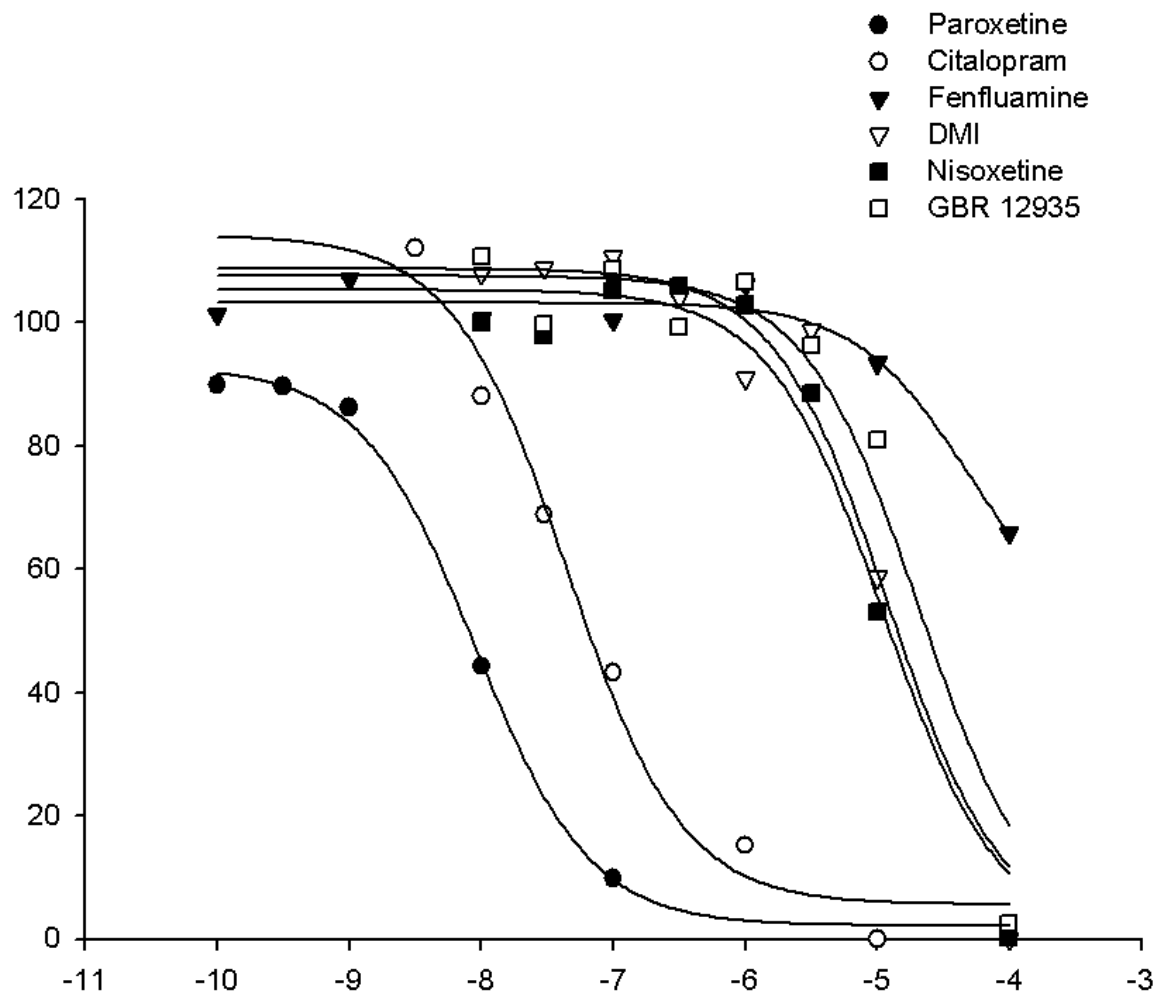


Figure 5

