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Presynaptic adaptive responses to constitutive versus adult pharmacologic inhibition of serotonin uptake

ABSTRACT

Many antidepressants are believed to relieve depressed mood and excessive anxiety by inhibiting the reuptake of serotonin so as to cause increases in extracellular serotonin. This homeostatic alteration is thought to underlie further adaptive processes – which have not been fully clarified – that together constitute the cellular mechanisms of current antidepressant therapy. Here, we review the literature on presynaptic adaptive responses to chronic antidepressant treatment, focusing on alterations in serotonin transporter (SERT) expression, extracellular and intracellular serotonin levels, and serotonergic innervation. We contrast this with studies on constitutive loss of SERT gene expression. A partial genetic reduction in SERT expression results in modest increases in extracellular serotonin, while the total absence of SERT is associated with substantial increases in extracellular serotonin, decreases in intracellular serotonin, and a reduction in serotonin immunopositive cell bodies and axons in the dorsal raphe and hippocampus, respectively. Adaptive changes in SERT protein levels and extracellular and intracellular serotonin concentrations following many different regimens of chronic antidepressant administration were found to be more variable, often falling in between those resulting from partial and complete genetic ablation of SERT. This might reflect incomplete pharmacologic inhibition of SERT and the wide variety of drug administration paradigms utilized. The microdialysis literature, in particular, suggests that it is difficult to conclude that chronic antidepressant

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treatment reliably causes elevated extracellular serotonin. However, with the exception of immunocytochemical studies, which were few and reported opposing findings, presynaptic adaptations occurring in response to antidepressants were qualitatively similar to those resulting from constitutive reductions in SERT. Thus, these particular presynaptic neuroadaptive responses by themselves are not sufficient to explain paradoxical increases in trait anxiety that accompany constitutive reductions in SERT expression in mice, rats, and humans.

INTRODUCTION

Long-term reduction in the recapture of serotonin (5-HT) from the extracellular space by the serotonin transporter (SERT) is a powerful adaptive force and the mechanisms of many antidepressants are believed to involve chronic SERT inhibition. However, changes in emotionally related behavior in response to reduced uptake occurring over different periods of life are dichotomous. Pharmacologic inhibition of SERT decreases anxiety and depressive symptoms in the subset of adult patients who respond to commonly prescribed antidepressants. By contrast, constitutive reductions in SERT expression occurring throughout development are correlated with increased anxiety-related behavior in mice (Holmes *et al.*, 2003a, 2003b) and heightened personality traits associated with negative emotionality in humans (Greenberg *et al.*, 2000; Lesch *et al.*, 1996; Schinka *et al.*, 2004; Sen *et al.*, 2004).

In this chapter, we examine the impact of genetically driven SERT deficiency on the neurochemistry of the presynaptic serotonergic system in rodent models. We compare this scenario to the relatively larger and more complex picture of presynaptic serotonergic responses to chronic antidepressant administration in adult animals. We focus on adaptations in the expression of SERT itself, effects on extracellular and intracellular serotonin levels, and changes in serotonergic neuronal architecture. Along with numerous studies published by many different authors, we integrate the results of our own studies on serotonin neurochemistry in SERT-deficient mice and present new data on the effects of reduced SERT expression on serotonergic innervation of the adult hippocampus. Our goal in analyzing and comparing these two bodies of literature is to determine whether differences in the effects of constitutive versus adult pharmacologic uptake inhibition on presynaptic neurochemistry provide a basis for understanding divergent phenotypic outcomes.

SEROTONIN TRANSPORTER EXPRESSION

Three different groups of investigators have produced mice with constitutive decreases in SERT expression (Bengel *et al.*, 1998; Lira *et al.*, 2003; Zhao *et al.*, 2006). SERT-deficient mice have been used to study the effects of reduced serotonin uptake on pre- and postsynaptic function with the goal of increasing information about the role of serotonin in modulating a number of important behaviors. Targeted disruption of exon 2, which contains the SERT gene start codon, results in a gene dose-dependent loss of full-length SERT mRNA (Bengel *et al.*, 1998). A truncated SERT message continues to be transcribed, which is translated into a non-functional and abnormally trafficked protein (Ravary *et al.*, 2001). In our initial study, SERT binding sites were assessed by quantitative autoradiography using [^{125}I]RTI-55, a cocaine analog with high affinity for SERT (Andrews *et al.*, 1996; Bengel *et al.*, 1997), in mice on a mixed 129 × CD-1 background (Bengel *et al.*, 1998). A 50% reduction in SERT density occurred in SERT+/− mice across a wide range of brain regions, while the complete absence of SERT was observed in null mutant mice (Figure 1.1; Bengel *et al.*, 1998; Perez *et al.*, 2006). A 50% decrease and a complete lack in SERT binding have also been reported in the CA3 region of the hippocampus of SERT+/− and SERT−/− mice, respectively, using the radiolabeled antidepressant [^{3}H]cyanoimipramine (Montanez *et al.*, 2003). These mice were produced from the same founders as mice initially reported by Bengel and coworkers, but they had been bred onto a C57BL/6J background. Sora and coworkers reported on SERT labeled by [^{3}H]paroxetine in SERT-deficient mice in the C57BL/6J background that had been cross-bred with mice deficient in the dopamine transporter (DAT; Sora *et al.*, 2001). Here, DAT+/+ × SERT+/− mice showed a ∼50% decrease in SERT binding and SERT was not detected in DAT+/+ × SERT−/− mice. In mice generated independently using a similar gene inactivation strategy but bred onto a 129S6/SvEv background, autoradiography of brain sections with [^{125}I]DAM (5-iodo-2–[[2-2-[dimethylamino)methyl]phenyl]thio]benzyl alcohol) showed undetectable levels of SERT in SERT−/− mice compared to wildtype littermates (Lira *et al.*, 2003). Zhao *et al.* used a different gene targeting strategy to produce mice with a disruption of the C-terminus of the SERT gene in a 129S5 × C57BL/6J hybrid background (2006). Homozygous mutant mice also showed a complete loss of SERT analyzed by saturation binding with [^{3}H]citalopram in brain tissue homogenates (Zhao *et al.*, 2006).

The selectivity of two additional SERT ligands, AFM ([^{3}H]2-[2-(dimethylaminomethylphenylthio)]-5-fluoromethylphenylamine) and

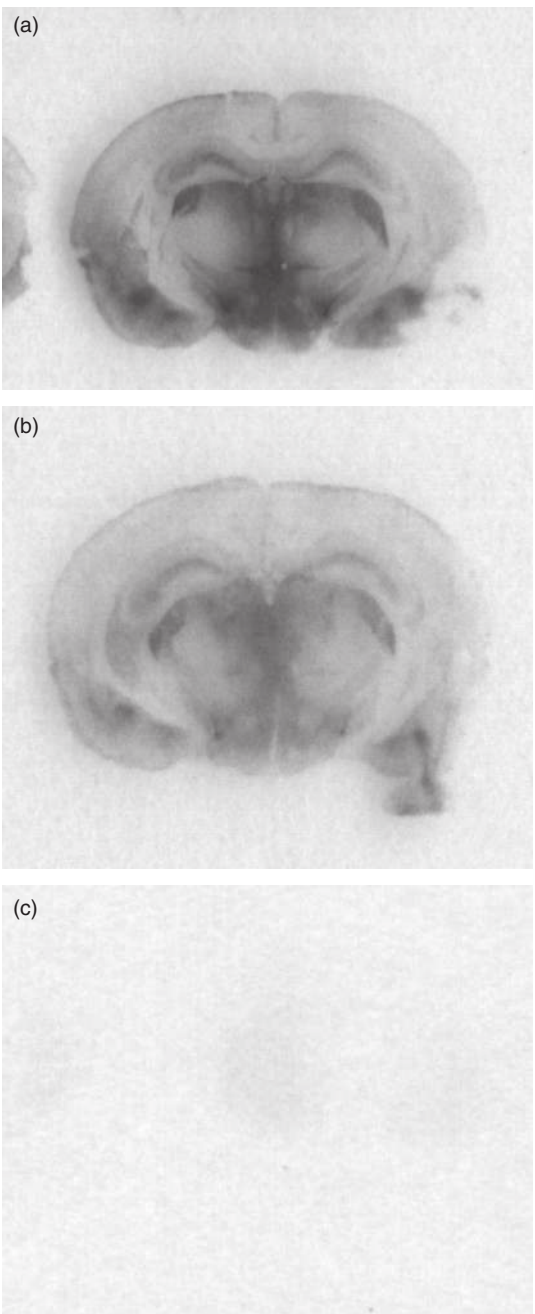

Figure 1.1 Serotonin transporter autoradiography in mice with constitutive Reductions in SERT. The cocaine analog [^{125}I]RTI-55 was used

DASB ([^{3}H]3-amino-4-[2-(dimethylaminomethylphenylthio)]benzonitrile), recently developed for positron emission tomography (PET) imaging, has been investigated by autoradiography in mice lacking one or both copies of the SERT gene (Li *et al.*, 2004). High densities of [^{3}H]AFM and [^{3}H]DASB binding were observed in the hippocampus, thalamus, raphe nuclei, and locus coeruleus of SERT+/+ mice. SERT+/− mice exhibited reduced binding to ∼50% of that detected in wildtype mice. As anticipated, no binding was observed for either ligand in any of the brain regions analyzed in SERT−/− mice (Li *et al.*, 2004). Thus, there is agreement among published studies that intact serotonin transporter protein labeled by many different radioligands is reduced in a gene dose-dependent manner in SERT-deficient mice generated by alternate strategies in a variety of genetic backgrounds.

Many studies have been conducted that have assessed the effects of long-term pharmacologic inhibition of serotonin reuptake on SERT protein levels. Here, the majority of studies conclude that a reduction in SERT occurs after chronic administration of selective serotonin reuptake inhibitors (SSRIs) to mice (Hirano *et al.*, 2005; Mirza *et al.*, 2007) or rats (Benmansour *et al.*, 1999, 2002; Brunello *et al.*, 1987; Gould *et al.*, 2006, 2007; Horschitz *et al.*, 2001; Kovachich *et al.*, 1992; Pineyro *et al.*, 1994; Rossi *et al.*, 2008; Watanabe *et al.*, 1993). In these studies, SERT protein levels or binding sites have been evaluated by Western blot and immunochemistry or quantitative autoradiography, respectively. Decreases in SERT are reportedly widespread, occurring in subregions of the hippocampus (CA2, CA3 and dentate gyrus), the basolateral and central nuclei of the amygdala, (fronto)parietal cortex, perirhinal cortex, striatum, thalamus, and midbrain (Benmansour *et al.*, 1999, 2002; Gould *et al.*, 2006, 2007; Hirano *et al.*, 2005; Kovachich *et al.*, 1992; Mirza *et al.*, 2007; Pineyro *et al.*, 1994; Rossi *et al.*, 2008; Watanabe *et al.*, 1993). From these data, it appears that reductions in SERT occur in

Caption for Figure 1.1 (cont.)
to label serotonin transporter (SERT) binding sites in wildtype mice (a), mice lacking one intact copy of the SERT gene (b), and mice lacking both intact SERT gene copies (c). Representative coronal sections are shown at the level of the rostral hippocampus. Densitometric analyses (Bengel *et al.*, 1998; Perez and Andrews, 2005) have shown that SERT binding is reduced by ∼50% in all brain regions in SERT+/− mice compared to SERT+/+ mice, and is not detected in any brain region in SERT−/− mice.

projection networks originating from both the dorsal and median raphe nuclei.

However, not all reports show decreased SERT levels in response to chronic SSRI administration. An early study by Hrdina and Vu (1993) reported that chronic treatment of rats with fluoxetine resulted in an increase in SERT labeling in cortical areas and the CA1 region of hippocampus, and a smaller increase in the superior colliculus. Some of these are the same regions where others have reported reductions in SERT protein levels. A number of studies have reported no change in SERT following long-term administration of the SSRI citalopram to rats (Cheetham *et al.*, 1993; Gobbi *et al.*, 1997; Gould *et al.*, 2006; Graham *et al.*, 1987; Kovachich *et al.*, 1992), with the exception of one study where a decrease in SERT levels following citalopram was observed (Brunello *et al.*, 1987). Interestingly, two of these same studies also noted reductions in SERT following chronic treatment with a different SSRI, sertraline (Gould *et al.*, 2006; Kovachich *et al.*, 1992). Interestingly, tianeptine, which is reported to be a serotonin reuptake enhancer, was also found to decrease SERT binding in cortex and hippocampus (Watanabe *et al.*, 1993).

In addition to in vivo experiments, the regulation of SERT by antidepressant treatment has been investigated in in vitro systems (Iceta *et al.*, 2007; Lau *et al.*, 2008). Iceta and coworkers studied an enterocyte-like cell line that natively expresses human SERT (2007). Their results showed that four consecutive days of treatment with fluoxetine reduced plasma membrane SERT without altering total SERT protein or mRNA levels. Similarly, in a recent study by Lau *et al.* (2008), citalopram treatment of human SERT-transfected HEK293 kidney cells resulted in the time-dependent translocation of SERT to intracellular compartments. Following treatment of murine stem cell-derived serotonergic neurons ($1C11^{5HT}$ cells) with citalopram, SERT was similarly internalized, in addition to being relocated from neurite extensions to cell bodies, without affecting cell morphology or neurite outgrowth (Lau *et al.*, 2008). Citalopram-free medium initiated the movement of SERT from the soma back to neurites and the same SERT reappeared on the cell surface, as evidenced by co-treatment with a protein synthesis inhibitor. In addition to providing new information about the mechanisms by which antidepressants modulate SERT, the results of these studies raise the possibility that earlier discrepancies regarding the effects of chronic antidepressant treatment on SERT expression may be due to methodological issues involving the labeling of SERT localized to different subcellular compartments. A number of intracellular

signaling mechanisms have been reported to regulate SERT surface expression, including protein kinase C (Qian *et al.*, 1997; Ramamoorthy *et al.*, 1998). Substrates and antagonists of SERT, such as serotonin and antidepressants, respectively, mediate PKC-dependent SERT phosphorylation and the redistribution of cell-surface SERT (Blakely *et al.*, 2005; Ramamoorthy and Blakely, 1999). Therefore, additional experiments will be needed to differentiate SERT localized to different subcellular compartments and to address how changes in specific populations of SERT occur in response to chronic antidepressant treatment, particularly in vivo.

A number of additional factors come into play when interpreting data from studies on antidepressant-induced regulation of SERT. These include the possibility that specific SSRIs (or tricyclic antidepressants) have different effects, as evidenced in studies performed by two different groups (Gould *et al.*, 2006; Kovachich *et al.*, 1992). In addition, the route of administration is a consideration. For instance, Hrdina and Vu (1993) and Gobbi *et al.* (1997) both administered fluoxetine to rats for 21 days; however, the drug was given intraperitoneally and perorally, respectively. Differences in routes of administration might underlie some of the conflicting data resulting from these two studies. An additional issue involves whether drug levels in preclinical studies reach steady-state human therapeutic serum concentrations. In rodents, the half-lives of many antidepressants are significantly shorter than in humans (Fredricson Overo, 1982; Hiemke and Hartter, 2000; Melzacka *et al.*, 1984). The use of osmotic minipumps circumvents problems associated with metabolism, and many investigators have incorporated the use of these devices for this reason (Benmansour *et al.*, 1999, 2002; Gould *et al.*, 2006, 2007; Hirano *et al.*, 2005; Koed and Linnet, 1997; Lesch *et al.*, 1993; Neumaier *et al.*, 1996; Pineyro *et al.*, 1994). Interestingly, the majority of studies that did not utilize osmotic minipumps reported increases (Hrdina and Vu, 1993) or no change (Cheetham *et al.*, 1993; Gobbi *et al.*, 1997; Graham *et al.*, 1987; Spurlock *et al.*, 1994; Swan *et al.*, 1997) in SERT protein levels following chronic SSRI treatment with the exception of two studies (Kovachich *et al.*, 1992; Mirza *et al.*, 2007), as opposed to consistent decreases in SERT protein or binding reported in studies employing minipump administration.

The time between the cessation of drug treatment and the measurement of SERT, otherwise known as the washout period, may also influence results. Moreover, it will be important to understand whether the effects of chronic antidepressant treatment cause global changes in SERT expression, similar to those occurring in genetic models, or

whether specific brain regions are modulated differently by pharmacologic inhibition of SERT. In summary, it seems that consensus has not been reached regarding the most appropriate methods for administering antidepressants, so as to determine more clearly how long-lasting inhibition of serotonin reuptake modulates serotonin transporter protein levels and dynamic regulation of subcellular SERT distribution in the brain.

At the level of transcription, reports on the regulation of SERT following long-term administration of antidepressants are inconsistent. A number of groups have reported no change in SERT mRNA levels after chronic administration of SSRIs to rats (Benmansour *et al.*, 1999; Koed and Linnet, 1997; Spurlock *et al.*, 1994; Swan *et al.*, 1997), or treatment of cells expressing human SERT (Iceta *et al.*, 2007), or murine stem cell-derived serotonergic neuron cells (Lau *et al.*, 2008). By contrast, Benmansour and colleagues found that 21 days of treatment with 10 mg/kg paroxetine via osmotic minipumps in rats had no effect on SERT mRNA levels in the median raphe; however, it resulted in a trend toward an increase in SERT mRNA in the dorsal raphe nucleus as quantified by in situ hybridization histochemistry (Benmansour *et al.*, 1999). In a later study, this group found that sertraline administration (7.5 mg/kg for 10 days) increased SERT mRNA levels by approximately 30% in the dorsal raphe, but these levels returned to baseline after 21 days of treatment (Benmansour *et al.*, 2002). Together, these two studies suggest that chronic antidepressant treatment regulates SERT transcription in the dorsal raphe nucleus, one of the main nuclei containing the serotonergic cell bodies that project to many areas of the forebrain. They also imply that changes in SERT mRNA may be transient, possibly explaining why no changes in SERT mRNA levels have been reported in other studies where intermediate time points were not investigated. Neumaier and coworkers also observed temporally related changes in SERT mRNA levels; however, in this study, chronic fluoxetine treatment (3 mg/kg/day) in rats via osmotic minipumps resulted in decreased SERT mRNA in the dorsal raphe after 7 days of treatment, with mRNA returning to control levels after 21 days of fluoxetine as determined by in situ hybridization (Neumaier *et al.*, 1996). Although Benmansour *et al.* and Neumaier *et al.* reported that the subchronic effects of antidepressant treatment on SERT mRNA levels changed in opposite directions, both studies point to the possibility of time-dependent alterations in SERT mRNA occurring after the initiation of antidepressant administration. In another study by Lesch and colleagues, a decrease in SERT mRNA levels (~30%) in the

rat midbrain raphe complex was observed by Northern blot following treatment with fluoxetine (2.5 mg/kg) via osmotic minipump for 21 days (Lesch *et al.*, 1993). These results conflict with the other studies described above. Thus, regulation of SERT at the transcriptional level by chronic inhibition of serotonin reuptake is not fully understood. Here, inconsistencies in the results of the studies discussed are not due to a lack of steady-state drug levels since osmotic minipumps were used to deliver drugs in all cases.

Tricyclic antidepressants have also been investigated with regard to the role they play in the regulation of SERT mRNA, and the data are similarly lacking in agreement. For example, following chronic treatment of rats with imipramine, SERT mRNA levels were reported to increase (Lopez *et al.*, 1994), to decrease (Lesch *et al.*, 1993), or not to change (Burnet *et al.*, 1994; Koed and Linnet, 1997; Spurlock *et al.*, 1994) compared to levels in animals treated with vehicle. Chronic treatment with the atypical antidepressant tianeptine resulted in decreased SERT mRNA (Kuroda *et al.*, 1994). The use of in situ hybridization versus Northern blotting cannot be correlated with upregulation, downregulation, or a lack of effect on SERT mRNA, suggesting that these disparities do not arise from methodological issues. The studies discussed here are by no means exhaustive with regard to reports on the regulation of SERT mRNA by long-term antidepressant administration. However, they highlight some of the discrepancies in this area and indicate that there is much to learn before we understand how pharmacologic inhibition of 5-HT reuptake regulates SERT at the level of transcription, in addition to protein levels and membrane trafficking.

EXTRACELLULAR SEROTONIN LEVELS

Antidepressants including SSRIs, mixed serotonin and norepinephrine reuptake inhibitors (SNRIs), and tricyclic antidepressants are thought to act by blocking the reuptake of serotonin and/or norepinephrine at their respective transporters. This inhibition is hypothesized to increase extracellular neurotransmitter levels, which results in the alleviation of anxiety and depressive symptoms in some patients by additional adaptive mechanisms that have yet to be fully elucidated. Studies in mice constitutively lacking both copies of the SERT gene support the idea that the loss of serotonin reuptake results in elevated extracellular levels of 5-HT. Compared to wildtype mice, dialysate 5-HT levels were increased in SERT−/− mice in frontal cortex, striatum (Mathews *et al.*, 2004; Shen *et al.*, 2004; Trigo *et al.*, 2007), and ventral

hippocampus (Whittington and Virag, 2006) as determined by in vivo microdialysis. Rats constitutively lacking SERT have also been reported to possess significantly increased amounts of dialysate 5-HT levels in the ventral hippocampus (Homberg *et al.*, 2007). Conversely, mice overexpressing SERT exhibit decreased extracellular 5-HT (Jennings *et al.*, 2006). Thus, constitutive absence of functional SERT protein results in augmented extracellular 5-HT levels in adult animals, while increased expression of SERT appears to diminish levels of extracellular 5-HT. On the contrary, chronic administration of antidepressants and, in particular, those purported to block the action of SERT have not resulted consistently in similar findings.

Many microdialysis studies have been carried out in rats to investigate the effects of chronic antidepressant treatment on dialysate 5-HT levels, with only one study having been conducted in mice (Gardier *et al.*, 2003). Table 1.1, which is organized by brain region, summarizes this literature and reveals the substantial disagreement that exists with regard to the effects of chronic antidepressants on dialysate 5-HT levels. For example, in the hippocampus, four studies reported increases (Gundlah *et al.*, 1997; Hajos-Korcsok *et al.*, 2000; Kreiss and Lucki, 1995; Wegener *et al.*, 2003), while the majority of studies carried out in this brain region found no change in basal dialysate 5-HT following long-term antidepressant administration (Bosker *et al.*, 1995a, 1995b; Gardier *et al.*, 2003; Hjorth and Auerbach, 1999; Invernizzi *et al.*, 1995; Keck *et al.*, 2005; Tachibana *et al.*, 2006). The most striking discrepancies are observed in studies using SSRIs, including citalopram (Gundlah *et al.*, 1997; Hjorth and Auerbach, 1994, 1999; Invernizzi *et al.*, 1995; Wegener *et al.*, 2003), fluoxetine (Kreiss and Lucki, 1995), fluvoxamine (Bosker *et al.*, 1995a, 1995b; Tachibana *et al.*, 2006), and paroxetine (Gardier *et al.*, 2003; Hajos-Korcsok *et al.*, 2000; Keck *et al.*, 2005). Chronic administration of tricyclic antidepressants (Gur *et al.*, 1999b; Hajos-Korcsok *et al.*, 2000; Newman *et al.*, 2000) and SNRIs (Gur *et al.*, 1999a, 2002a; Tachibana *et al.*, 2006) more consistently show no increase in hippocampal dialysate 5-HT levels.

In the hippocampus, discrepancies cannot be explained by the specific subregions investigated. For example, no changes in 5-HT levels after all classes of antidepressants have been observed in both the dorsal (Bosker *et al.*, 1995a, 1995b; Hjorth and Auerbach, 1994, 1999; Invernizzi *et al.*, 1995; Keck *et al.*, 2005; Tachibana *et al.*, 2006) and the ventral hippocampus (Gardier *et al.*, 2003; Gur *et al.*, 1999a, 1999b, 2002a; Newman *et al.*, 2000). Washout times are also not likely to be responsible for the discrepancies. The majority of hippocampal studies