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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.

I. 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 $\times$ 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+/+ $\times$ SERT+/− mice showed an $\sim$50% decrease in SERT binding and SERT was not detected in DAT+/+ $\times$ 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 $\times$ 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

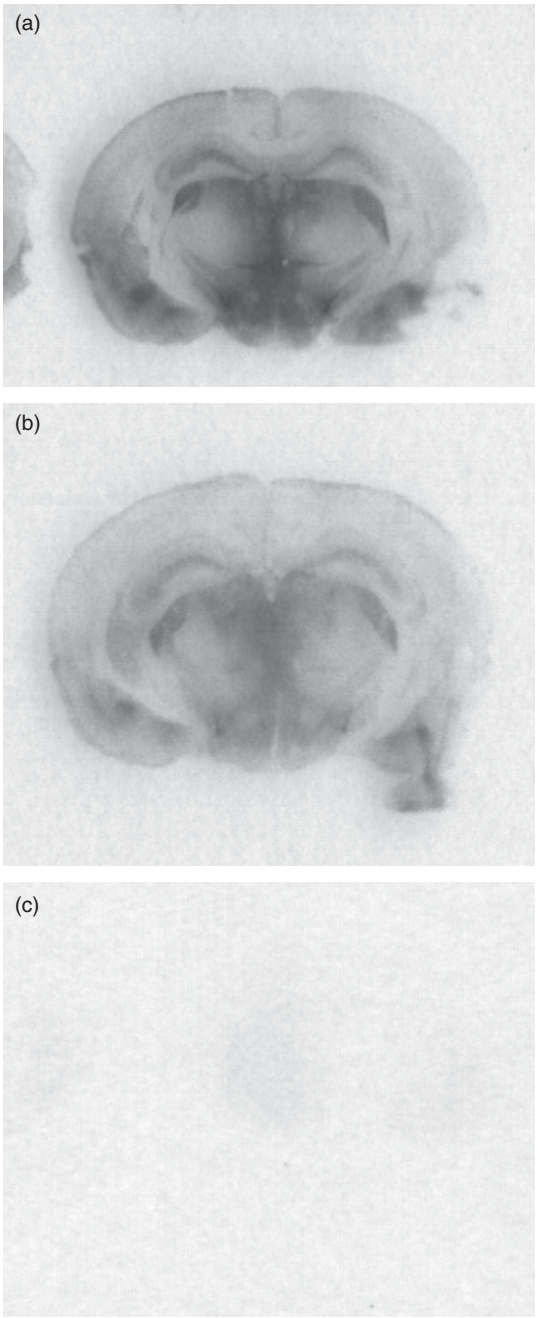

Figure 1.1 Serotonin transporter autoradiography in mice with constitutive Reductions in SERT. The cocaine analog [^{125}I]RTI-55 was used

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 neurons 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 ($\sim$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.

II. 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 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

Table 1.1.

Class	Drug	Dose (mg/kg/day)	ROA[1]	Days	Dialysate 5-HT[2]	Reference
Hippocampus						
SSRI	Citalopram	5 × 2	sc	14	nc	Hjorth and Auerbach (1994)
		10 × 2	sc	14	↑	Gundlah *et al.* (1997)
		10 × 2	sc	14	nc	Hjorth and Auerbach (1999)
		10 × 2	sc	14	nc	Invernizzi *et al.* (1995)
		20	minipump	21	↑	Wegener *et al.* (2003)
	Fluoxetine	15	ip	14	↑	Kreiss and Lucki (1995)
	Fluvoxamine	6.7	minipump	23	nc	Bosker *et al.* (1995b)
		30	oral	14	nc	Tachibana *et al.* (2006)
		30	oral	14	nc	Bosker *et al.* (1995a)
	Paroxetine	1	minipump	14	nc[a]	Gardier *et al.* (2003)
		5	oral	70	nc	Keck *et al.* (2005)
		5	sc	14	↑, nc[b]	Hajos-Korcsok *et al.* (2000)
TCA	Clomipramine	10	ip	28	nc	Gur *et al.* (1999b)
		10	minipump	28	nc	Newman *et al.* (2000)
	Desipramine	10	sc	14	nc	Hajos-Korcsok *et al.* (2000)
		15	ip	14	nc	Kreiss and Lucki (1995)
SNRI	Milnacipran	30	oral	14	nc	Tachibana *et al.* (2006)
	Venlafaxine	5	ip	28	nc	Gur *et al.* (1999a)
		5	minipump	28	nc	Gur *et al.* (2002a)

Table 1.1. (cont.)

Class	Drug	Dose (mg/kg/day)	ROA[1]	Days	Dialysate 5-HT[2]	Reference
Frontal cortex						
SSRI	Citalopram	5 × 2	sc	14	nc	Hjorth and Auerbach (1999)
		10	ip	14	↑	Golembiowska and Dziubina (2000)
		10 × 2	sc	14	nc	Gundlah *et al.* (1997)
		10 × 2	sc	14	nc	Hjorth and Auerbach (1999)
		20	minipump	13	↑	Ceglia *et al.* (2004)
		20	ip	14	↑, nc[c]	Arborelius *et al.* (1996)
	Fluoxetine	3	minipump	14	↑	Amargos-Bosch *et al.* (2005)
		3	minipump	14	↑, nc[d]	Hervas *et al.* (2001)
		5	ip	7	nc	Lifschytz *et al.* (2004)
		5	ip	12	↑	Newman *et al.* (2004)
		10	ip	11–12	↑	Gartside *et al.* (2003)
		10	ip	14	nc	Dawson *et al.* (2000)
		10	ip	14	nc	Dawson *et al.* (2002)
		10	ip	14	↑	Invernizzi *et al.* (1996)
		10	ip	14	↑, nc[e]	Johnson *et al.* (2007)
		10	oral	21	↑	Mitchell *et al.* (2001)
	Fluvoxamine	1	minipump	7	↑	Bel and Artigas (1993)
	Paroxetine	0.5 × 2	ip	14	nc	Malagie *et al.* (2000)
		1	minipump	14	nc[a]	Gardier *et al.* (2003)
		10	ip	14	↑	Owen and Whitton (2006)
		10	ip	21	↑	Owen and Whitton (2005)

TCA	Clomipramine	10	ip	14	↑	Owen and Whitton (2006)
		10	ip	21	↑	Owen and Whitton (2005)
		10	ip	28	↑	Gur *et al.* (1999b)
	Imipramine	4	minipump	14	↑	Bel and Artigas (1996)
		10	minipump	28	nc	Gur *et al.* (2002b)
SNRI	Duloxetine	6.25	oral	14	nc	Kihara and Ikeda (1995)
	Venlafaxine	5	ip	28	nc	Gur *et al.* (1999a)
		10	minipump	14	↑	Wikell *et al.* (2002)
NRI	Reboxetine	10	ip	14	↑	Owen and Whitton (2006)
		10	ip	21	↑	Owen and Whitton (2005)
		10	minipump	14	nc	Page and Lucki (2002)
		10 (13)[f]	minipump	14	nc	Invernizzi *et al.* (2001)
MAO	Tranylcypromine	0.5	minipump	14	↑	Ferrer and Artigas (1994)
Other	Tianeptine	5 × 2	ip	14	nc	Malagie *et al.* (2000)
	$LiCO_3$	0.2%[g]	oral	7	nc	Kitaichi *et al.* (2005)
Hypothalamus						
SSRI	Citalopram	50	oral	21	↑, nc[h]	Moret and Briley (1996)
	Fluoxetine	5	ip	7	nc	Lifschytz *et al.* (2004)
		5	ip	12	nc	Newman *et al.* (2004)
		10	ip	14	↑	Rutter *et al.* (1994)
	Paroxetine	10	ip	21	nc	Sayer *et al.* (1999)
TCA	Clomipramine	10	minipump	28	↑	Newman *et al.* (2000)
	Desipramine	10	ip	21	nc	Sayer *et al.* (1999)
	Imipramine	10	minipump	28	nc	Gur *et al.* (2004)

Table 1.1. (cont.)

Class	Drug	Dose (mg/kg/day)	ROA[1]	Days	Dialysate 5-HT[2]	Reference
SNRI	Venlafaxine	5	minipump	28	nc	Gur *et al.* (2002a)
Striatum						
SSRI	Fluoxetine	10	ip	14	↑	Rossi *et al.* (2008)
		15	ip	14	↑	Kreiss and Lucki (1995)
	Sertraline	10	minipump	21	nc	Rossi *et al.* (2008)
TCA	Desipramine	15	ip	14	↑	Kreiss and Lucki (1995)
NRI	Reboxetine	15	ip	14	nc	Sacchetti *et al.* (1999)
Raphe nuclei						
SSRI	Fluvoxamine	1	minipump	7	nc	Bel and Artigas (1993)
		30	oral	14	nc	Bosker *et al.* (1995a)
	Paroxetine	0.5 × 2	ip	14	↑	Malagie *et al.* (2000)
TCA	Imipramine	4	minipump	14	nc	Bel and Artigas (1996)
MAO	Tranylcypromine	0.5	minipump	14	↑	Ferrer and Artigas (1994)
SSRE	Tianeptine	5 × 2	ip	14	nc	Malagie *et al.* (2000)
Amygdala						
SSRI	Citalopram	∼20	minipump	14	nc	Bosker *et al.* (2001)

Notes:

[1]Route of administration.

[2](↑) Increase in dialysate 5-HT was reported; (nc) no change in dialysate 5-HT was reported.

[a]Study conducted in mice (129/Sv) using zero net flux as opposed to basal dialysate studies in rats.

[b]Increase only when drug was given 2× daily not when given 1× daily.

[c]Increase was observed 10–12 h but not 18–20 h after the end of treatment.

[d]Increase was observed without a 48 h washout but not with a 48 h washout.

[e]Increase in two experiments in study; change only approached significance in a third experiment.

[f]Stated dose was 10 mg/kg/day but other information provided in the paper indicated a dose equal to 13 mg/kg/day.

[g]Rats were given chow containing 0.2% $LiCO_3$.

[h]Increase was observed without a 24 h washout but not with a 24 h washout.

listed in Table 1.1 reported a minimum of 22 h for drug washout, with a few exceptions where drugs were either not washed out prior to microdialysis (Bosker *et al.*, 1995b; Wegener *et al.*, 2003), or the washout period was not specified (Keck *et al.*, 2005; Tachibana *et al.*, 2006). Because only four studies have reported increases in extracellular hippocampal 5-HT, it is difficult to determine whether a correlation can be made between these studies and the routes of administration employed. For example, while oral administration of drugs consistently failed to increase hippocampal 5-HT levels, those studies that did report elevations utilized both subcutaneous and intraperitoneal injections, as well as osmotic minipumps. A wide variety of drugs have been studied, but dose–response relationships have not been investigated specifically, perhaps with the exception of citalopram. Even with this drug, there does not appear to be a trend across studies towards a dose–effect relationship in the hippocampus (Gundlah *et al.*, 1997; Hjorth and Auerbach, 1994, 1999; Invernizzi *et al.*, 1995; Wegener *et al.*, 2003).

Papers reporting on dialysate levels of 5-HT in the frontal cortex after chronic antidepressant treatment are more numerous but similarly divided in their observations. Some groups reported no change in 5-HT levels after chronic citalopram (Gundlah *et al.*, 1997; Hjorth and Auerbach, 1994, 1999), while others found that SSRIs, such as citalopram (Arborelius *et al.*, 1996; Ceglia *et al.*, 2004; Golembiowska and Dziubina, 2000) or fluvoxamine (Bel and Artigas, 1993), cause increases in dialysate 5-HT in frontal cortex. Administration of paroxetine has been shown to cause no change (Gardier *et al.*, 2003; Malagie *et al.*, 2000) or to elevate (Owen and Whitton, 2005, 2006) frontal cortex concentrations of dialysate 5-HT. In this brain region, there is evidence that a dose–response effect may account for different responses to paroxetine, with increases observed only at higher drug doses. However, additional studies will be necessary to confirm this. Contradictory results pertaining to the frontal cortex have also been published regarding fluoxetine, as it reportedly increases (Amargos-Bosch *et al.*, 2005; Gartside *et al.*, 2003; Hervas *et al.*, 2001; Invernizzi *et al.*, 1996; Mitchell *et al.*, 2001; Newman *et al.*, 2004) or does not influence (Dawson *et al.*, 2000, 2002; Hervas *et al.*, 2001; Lifschytz *et al.*, 2004) cortical levels of 5-HT in dialysates.

Closer inspection of the data reveals a number of perplexing inconsistencies in microdialysis studies on the effects of chronic antidepressant treatment on dialysate 5-HT in frontal cortex. For example, in comparing the results reported by Dawson and coworkers (2000, 2002), who studied the same dose of fluoxetine, using the same route

of administration and rat strain as Gartside *et al.* (2003) and Invernizzi and coworkers (1996), only the latter two studies reported increases in cortical 5-HT levels. Another study found that after using the same dose and route of administration of fluoxetine for the same duration to the same rat strain as Amargos-Bosch *et al.* (2005), 5-HT levels were only increased if a 48-h drug washout period was omitted (Hervas *et al.*, 2001). These two examples highlight how discrepant results can occur in separate laboratories even when similar experimental protocols are utilized. A recent and particularly pertinent example by Johnson and coworkers (2007) highlights similar issues. Two experiments in this study showed significant increases in basal dialysate 5-HT levels in the frontal cortex after chronic fluoxetine administration, while a third, using the same treatment regimen, did not reach significance (Johnson *et al.*, 2007).

Therefore, we cannot definitively conclude from the microdialysis literature that chronic fluoxetine increases extracellular levels of 5-HT in frontal cortex. Furthermore, even though a range of doses has been used in different studies on fluoxetine, changes in cortical levels of 5-HT were not correlated with dose. Although it may be tempting to conclude that washout periods have an effect on neurotransmitter levels, as suggested by the work of Hervas and colleagues (2001), both increases (Johnson *et al.*, 2007; Mitchell *et al.*, 2001; Newman *et al.*, 2004) and a lack of change (Dawson *et al.*, 2000, 2002; Johnson *et al.*, 2007; Lifschytz *et al.*, 2004) have been observed after washout periods of at least 24 h.

Conflicting observations regarding 5-HT levels in frontal cortex are not restricted to SSRIs. In rats, chronic clomipramine administration was reported to result in increased dialysate levels of 5-HT in frontal cortex (Gur *et al.*, 1999b; Owen and Whitton, 2005, 2006), whereas administration of a different tricyclic antidepressant, imipramine, has (Bel and Artigas, 1996) or has not (Gur *et al.*, 2002b) produced similar results. Treatment with duloxetine, an SNRI, resulted in increased frontal cortex levels of 5-HT (Kihara and Ikeda, 1995); however, a different SNRI, venlafaxine, reportedly had no effect (Gur *et al.*, 1999a) or elevated (Wikell *et al.*, 2002) frontal cortex 5-HT in the extracellular space. Similarly, chronic exposure to the norepinephrine reuptake inhibitor (NRI) reboxetine has both failed to elicit changes (Invernizzi *et al.*, 2001; Page and Lucki, 2002) and to induce increases (Owen and Whitton, 2005, 2006) in frontal cortex 5-HT dialysate levels. Studies using chronic regimens of atypical antidepressant drugs are similarly inconsistent. The monoamine oxidase inhibitor (MAOI) tranylcypromine increased dialysate 5-HT in frontal cortex (Ferrer and Artigas,

1994), while tianeptine (Malagie *et al.*, 2000) or $LiCO_3$ (Kitaichi *et al.*, 2005) failed to increase dialysate 5-HT levels in this brain region.

Thus, reports on dialysate 5-HT levels in the frontal cortex following chronic administration of antidepressants are not consistent. With the possible exception of paroxetine, there does not appear to be a correlation between dose and the effects on cortical 5-HT. Although drugs given subcutaneously did not increase dialysate 5-HT, the majority of studies utilizing intraperitoneal injections or osmotic minipumps observed elevated 5-HT. Oral drug administration resulted in conflicting results with respect to changes in dialysate 5-HT in frontal cortex. Too few studies have examined the influence of SNRIs, MAOIs or other classes of antidepressants (aside from SSRIs) on 5-HT levels in the cortex to draw conclusions regarding the effects of these drugs. Notably, frontal cortex was the brain region most often studied, yet the body of literature on this brain region is also the most divided.

Investigation of 5-HT levels in the hypothalamus in rats after chronic SSRI treatment has similarly resulted in contradictory outcomes. Several groups have reported no changes in basal levels of 5-HT (Lifschytz *et al.*, 2004; Moret and Briley, 1996; Newman *et al.*, 2004; Sayer *et al.*, 1999), although two groups have observed elevations in hypothalamic 5-HT (Moret and Briley, 1996; Rutter *et al.*, 1994). Furthermore, treatment with the tricyclic antidepressants imipramine (Gur *et al.*, 2004) or desipramine (Sayer *et al.*, 1999) did not change 5-HT levels in hypothalamus, while clomipramine was reported to increase dialysate 5-HT (Newman *et al.*, 2000). Only one SNRI, venlafaxine, has been studied and it was found to have no effect on dialysate hypothalamic 5-HT (Gur *et al.*, 2002a). With the exception of the study by Moret and Briley (1996), all of the papers pertaining to hypothalamus utilized a minimum of 24 h for drug washout. Based on these relatively few studies and the discrepant results in the hypothalamus, it is difficult to distinguish trends with regard to route of administration.

Only a handful of studies have examined dialysate levels of 5-HT in other brain regions including striatum, raphe nuclei, and amygdala in response to chronic antidepressant treatment. In striatum, levels of dialysate 5-HT were increased after fluoxetine (Kreiss and Lucki, 1995; Rossi *et al.*, 2008), but did not change following sertraline treatment (Rossi *et al.*, 2008). Similarly, while desipramine significantly increased striatal 5-HT (Kreiss and Lucki, 1995), the NRI reboxetine did not (Sacchetti *et al.*, 1999). Dialysate 5-HT levels in the raphe nuclei did not change after chronic fluvoxamine (Bel and Artigas, 1993; Bosker *et al.*, 1995a) or imipramine (Bel and Artigas, 1996), but were reported to rise following

paroxetine (Malagie *et al.*, 2000). Likewise, the MAOI tranylcypromine increased dialysate 5-HT in the raphe nuclei (Ferrer and Artigas, 1994), but the atypical antidepressant tiantepine failed to elevate 5-HT in this brain region (Malagie *et al.*, 2000). A lone study to examine dialysate 5-HT in the central nucleus of the amygdala observed no changes after chronic citalopram (Bosker *et al.*, 2001).

It is more often than not accepted that chronic antidepressant treatment and, in particular, long-term administration of SSRIs results in elevated levels of extracellular serotonin in brain regions important for the modulation of emotional behavior, such as the frontal cortex, hippocampus and hypothalamus. The analysis presented above demonstrates, however, that we are not yet in a position to draw this conclusion without ignoring almost half of the studies that indicate otherwise. Moreover, consideration of specific drugs, classes of drugs, and washout periods do not account for discrepant conclusions. Although a slight trend for subcutaneous injection and oral administration of drugs to fail to elicit changes in dialysate 5-HT across brain regions is noted, results from studies employing osmotic minipumps and intraperitoneal injections were evenly divided with regard to alterations in 5-HT levels. Future studies would need to compare routes of administration of antidepressants to conclude what influence this has on dialysate 5-HT levels determined by microdialysis.

One possible factor contributing to these variable outcomes involves issues surrounding in vivo recovery of 5-HT and the effect that reduced transport has on this factor. There are a number of quantitative microdialysis methods that have been developed (Kehr, 1993; Lonnroth *et al.*, 1987; Parsons and Justice, 1994) and utilized (Chefer *et al.*, 2006; Olson Cosford *et al.*, 1996) that attempt to account for the fact that recovery of neurotransmitters across microdialysis membranes is less than 100%. Furthermore, changes in the reuptake of neurotransmitters from the extracellular space, such as that occurring in response to antidepressant administration, are hypothesized to alter neurotransmitter concentration gradients in the tissue and, therefore, the flux (concentration per unit area per unit time) of neurotransmitter crossing the dialysis membrane.

We have used the method of zero net flux to correct for changes in the in vivo recovery of 5-HT theorized to occur as a result of reduced uptake in SERT-deficient mice (Mathews *et al.*, 2004). This method is based on the principle that when two solutions, such as the extracellular fluid in the brain and the artificial cerebrospinal fluid perfused into a microdialysis probe, are in contact across a semi-permeable

membrane, no net diffusion will occur between the two when they contain equal concentrations of the target analyte (e.g. 5-HT). Thus, by perfusing different concentrations of 5-HT into the dialysis probe and determining how the concentrations of 5-HT in the dialysates change, extracellular neurotransmitter concentrations can be estimated that are corrected for in vivo probe recovery. In practice, different discrete concentrations of 5-HT perfused into the microdialysis probe (C_{in}) are related to the measured concentrations of 5-HT in the dialysate (C_{out}). The differences between the two are calculated ($C_{in} - C_{out}$) and plotted on the *y*-axis against C_{in} on the *x*-axis. Linear regression yields the concentration where no net diffusion occurs, which is the point where the regression line crosses the *x*-axis. The slope of the regression line is the extraction fraction (E_d), which estimates in vivo probe recovery. Using this method, we have been able to detect as little as 1–2-fold increases in extracellular 5-HT in frontal cortex and striatum in SERT+/− mice compared to SERT+/+ mice that are not evident in basal dialysate 5-HT levels uncorrected for in vivo recovery (Mathews *et al.*, 2004).

Zero net flux has only been employed in one study on the effects of chronic antidepressant treatment on extraneuronal 5-HT (Gardier *et al.*, 2003). Here, mice were administered 1 mg/kg/day paroxetine for 14 days by osmotic minipump. The authors reported no differences in extracellular 5-HT levels corrected for in vivo recovery in the hippocampus or frontal cortex. This dose of paroxetine has been shown to result in mouse plasma drug levels comparable to therapeutic levels observed in human patients (Hirano *et al.*, 2005). It also significantly decreases SERT binding sites within both the cerebral cortex and the hippocampus of mice as measured by autoradiography (Hirano *et al.*, 2005). The only difference between these two studies that might explain the lack of change in extracellular 5-HT levels was that Hirano *et al.* administered paroxetine by osmotic minipump for 21 days (2005), as opposed to a shorter 14-day regimen used by Gardier *et al.* (2003). Future studies using a longer duration administration might be useful for determining whether alterations in extracellular 5-HT occur in response to this dose of paroxetine.

As a whole, the antidepressant microdialysis literature suggests that additional work will need to be done to ascertain more fully the effects of long-term inhibition of reuptake on extracellular 5-HT levels. Additionally, it may be beneficial to use quantitative microdialysis, as well as other in vivo neurochemical methods such as voltammetry methods (Stuart *et al.*, 2004; Wightman, 2006), to determine changes in extracellular 5-HT levels following chronic administration of SSRIs

and other antidepressants, as well as alterations occurring in response to constitutive deletions in SERT. Direct comparisons will allow assessment of the extent to which extracellular 5-HT is elevated in response to pharmacologic inhibition of SERT versus intermediate and complete SERT gene inactivation. We found no differences in extracellular levels of striatal dopamine in SERT-deficient mice (Mathews *et al.*, 2004); however, future studies might also benefit from investigating extracellular norepinephrine levels in SERT-deficient mice. Chronic administration of desipramine, which acts primarily as an inhibitor at the norepinephrine transporter, was reported to increase dialysate 5-HT in the striatum (Kreiss and Lucki, 1995). Thus, interactions between the norepinephrine and the 5-HT neurotransmitter systems may occur and underlie some of the complex mechanisms involved in the efficacy of antidepressants from different classes (Szabo *et al.*, 1999).

III. BRAIN TISSUE SEROTONIN LEVELS

The consequences of reducing 5-HT reuptake into presynaptic neurons via loss of SERT expression versus long-term pharmacologic inhibition of SERT have also been explored by determining concentrations of 5-HT in brain tissue. Barring a few exceptions, the data are in agreement that both genetic and pharmacologic reductions in SERT activity result in decreased 5-HT tissue levels. However, there is disagreement concerning the extent to which 5-HT is reduced following pharmacologic inhibition of 5-HT reuptake. Tissue 5-HT levels, which mainly reflect the combination of vesicular and cytoplasmic intracellular neurotransmitter pools, have been determined in a number of different studies on SERT-deficient mice. We originally reported that inactivation of one copy of the SERT gene has no effect on total tissue 5-HT content in frontal cortex, hippocampus, striatum, brain stem, and hypothalamus (Bengel *et al.*, 1998; Numis *et al.*, 2004) (Figure 1.2). By contrast, complete loss of SERT expression leads to 60–80% decreases in 5-HT in these brain regions. Fabre and colleagues (2000) reported similar magnitude reductions in 5-HT levels in cerebral cortex, hippocampus, striatum, and brain stem in SERT−/− mice. A recent study by Kim and coworkers (2005) confirmed no differences between brain tissue 5-HT levels in SERT+/+ and SERT+/− mice, but reported a slightly larger range of decreases in tissue 5-HT in SERT−/− mice to 40–70% of the levels measured in SERT+/+ mice. Tissue 5-HT levels have also been determined in BDNF+/+ × SERT−/− mice (BDNF, brain-derived neurotrophic factor; Ren-Patterson *et al.*, 2006). Here, 5-HT was decreased by

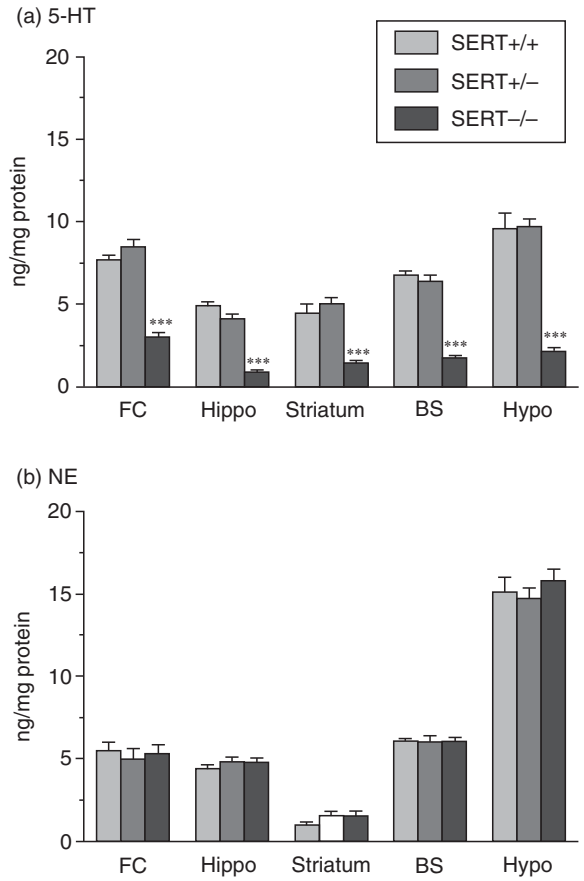

Figure 1.2 Tissue serotonin (a) and norepinephrine (b) concentrations in SERT-deficient mice determined by high performance liquid chromatography with electrochemical detection. FC, frontal cortex; Hippo, hippocampus; BS, brain stem; Hypo, hypothalamus. Probabilities are identified as $^{***}p<0.001$ for differences from SERT+/+ mice.

60–80% in hippocampus, hypothalamus, and brain stem and 50–65% in the striatum compared to BDNF+/+ × SERT+/+ mice bearing normal expression levels of both genes. In contrast to the association between reduced SERT expression and decreased tissue 5-HT levels, a recent paper by Jennings and colleagues reported that transgenic mice over-expressing the human serotonin transporter also show decreases in tissue 5-HT on the order of 15–35% in cortex, midbrain, brainstem, hippocampus, and hypothalamus compared to wildtype mice (Jennings

et al., 2006). Thus, both genetic deletion of SERT as well as increased expression of SERT may lead to reduced intracellular 5-HT, although the mechanisms that underlie changes in these two model systems may not be the same.

Statistically significant reductions in the major metabolite of 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), have also been found in SERT−/− but not SERT+/− mice. In the same brain regions, 5-HIAA levels are generally reduced to a lesser extent compared to 5-HT in response to genetic deletion of SERT, whereby reductions in 5-HIAA ranged from 30 to 50% in SERT−/− mice (Bengel *et al.*, 1998; Fabre *et al.*, 2000; Kim *et al.*, 2005) and 10–50% in BDNF+/+ × SERT−/− mice (Ren-Patterson *et al.*, 2006). Tissue 5-HIAA levels in mice over-expressing SERT were not significantly different from those in wildtype mice (Jennings *et al.*, 2006). Tissue norepinephrine (NE) levels have also been measured in SERT-deficient mice (Numis *et al.*, 2004; Tjurmina *et al.*, 2002). Here, no differences in NE levels were observed in frontal cortex, hippocampus, hypothalamus, striatum, and brain stem in SERT+/− or SERT−/− mice (Figure 1.2; Numis *et al.*, 2004). Moreover, plasma and adrenal gland NE levels in SERT+/− and SERT−/− mice were also unaltered compared to levels in SERT+/+ mice (Tjurmina *et al.*, 2002).

Data regarding the effects of chronic antidepressant treatment on brain 5-HT levels are more variable. In an early study, mice receiving intraperitoneal fluoxetine injections for 14 days failed to show changes in whole brain 5-HT levels (Hwang *et al.*, 1980). However, this study also reported that the same fluoxetine regimen reduced 5-HIAA levels by 50% compared to control animals. Also, long-term treatment of rats with the SSRIs paroxetine or sertraline via osmotic minipump for 21 days has been reported to have no effect on tissue levels of 5-HT or 5-HIAA in the hippocampus, even when accompanied by a reduction in SERT binding by 80–90% (Benmansour *et al.*, 1999). In contrast, a number of other studies carried out in rats came to the alternate conclusion that chronic treatment with SSRIs results in decreased tissue 5-HT levels (Caccia *et al.*, 1992; Durand *et al.*, 1999; Hrdina, 1987; Nakayama *et al.*, 2003). Caccia *et al.* and Hrdina each reported that a 21-day regimen of fluoxetine given by intraperitoneal injections is associated with 5-HT levels in cortex that are decreased by 45% and 30%, respectively. Serotonin levels were also investigated in the hippocampus of rats treated for 21 days with the SSRIs fluoxetine or sertraline. Here, tissue 5-HT levels were decreased by 30–40% (Caccia *et al.*, 1992; Nakayama *et al.*, 2003). Moreover, Durand and coworkers reported a decrease in hypothalamic 5-HT and 5-HIAA levels in two different

strains of rats receiving intraperitoneal fluoxetine for 21 days (Durand *et al.*, 1999). In cortex, hippocampus, and striatum, 5-HIAA levels were reportedly reduced by 10–35% in response to chronic administration of SSRIs (Hrdina, 1987; Nakayama *et al.*, 2003), whereas Caccia and colleagues observed larger decreases (50–60%) in 5-HIAA levels in cortex and hippocampus (Caccia *et al.*, 1992).

Therefore, the majority of studies suggest that chronic SSRI treatments result in reductions in intracellular 5-HT levels, which is similar to the effects of complete genetic loss of SERT. Decreases in 5-HIAA levels also occur regardless of whether reuptake is inhibited pharmacologically or genetically. Across the board, decreases in brain tissue 5-HT levels appear to be slightly higher and to occur more consistently in SERT−/− mice than in rodents receiving repeated antidepressant treatment. As suggested by the data on adaptive changes in SERT protein and extracellular 5-HT levels, variability in the effects of antidepressant on intracellular 5-HT levels may reflect incomplete inhibition of SERT, as a result of different drug doses and routes of administration. As such, the magnitude of adaptation may fall somewhere between that occurring in response to partial versus complete genetic ablation of SERT.

IV. SEROTONIN SYSTEM ARCHITECTURE

In addition to investigating the aspects of presynaptic adaptation discussed above, we have begun to examine the effects of constitutive reductions in SERT expression on serotonergic innervation of the forebrain. We have hypothesized that the decreases in total tissue 5-HT levels in SERT−/− mice described above (Figure 1.2) reflect a reduced vesicular pool of intracellular 5-HT that arises from the loss of reuptake of 5-HT by SERT (Bengel *et al.*, 1998; Kim *et al.*, 2005). However, other studies have shown that constitutive loss of SERT has the ability to alter the architecture of the brain, including the serotonin system, during development (Altamura *et al.*, 2007; Lira *et al.*, 2003; Persico *et al.*, 2003; Salichon *et al.*, 2001). Thus, we reasoned that lower tissue 5-HT levels might also be accounted for by decreased serotonergic axonal innervation of the forebrain.

Using immunocytochemistry, we have labeled 5-HT-containing axons with a polyclonal antibody against 5-HT (ImmunoStar, Inc., Hudson, WI) and quantified axon numbers in subregions of the hippocampus with respect to genotype in SERT-deficient mice. Specifically, serotonergic neuronal innervation and morphology were examined in 3–4-month-old female SERT-deficient mice in a mixed CD1 × 129S6/SvEv

background (n=6 SERT+/+, n=5 SERT+/− and n=5 SERT−/−). Axon quantification was performed using a Zeiss bright field microscope and Zeiss KS400 software (Carl Zeiss, Inc., Thornwood, NY) as described previously (Donovan *et al.*, 2002; Luellen *et al.*, 2006, 2007; Mamounas *et al.*, 1995). Dark field photomicrographs were selected to best represent the mean axon numbers determined by digital imaging for each treatment group. The brain regions of interest (ROIs) included the stratum radiatum layer of CA1, CA2 and CA3, the molecular layer of dentate gyrus (DG) and the granular layer of dentate gyrus (GrDG) of hippocampus. These regions were selected for study based on their innervation by the serotonin system and their involvement in the modulation of mood and anxiety-related behavior (Murphy *et al.*, 2004).

Analysis of variance revealed overall significant differences with respect to genotype in CA2 [$F(2,40)=14.8$; $p<0.001$], CA3 [$F(2,38)=3.4$; $p<0.05$], DG [$F(2,38)=3.5$; $p<0.05$], and GrDG [$F(2,38)=7.0$; $p<0.01$] regions of the hippocampus. Compared to SERT+/+ mice, mice with a 50% reduction in SERT expression showed no significant changes in 5-HT axon numbers in any of the hippocampal subregions studied, with the exception of a trend toward a significant increase in fibers in DG ($p=0.06$) (Figure 1.3A, 3B and 3D). By contrast, 35–70% decreases in axon numbers were observed in CA2 ($p<0.001$), CA3 ($p<0.05$) and GrDG ($p<0.01$) regions of hippocampus in mice completely lacking SERT (Figure 1.3A, 3C and 3D). Thus, the loss of serotonin reuptake occurring throughout development has the ability to alter serotonergic innervation in the adult hippocampus. Furthermore, decreased tissue levels of 5-HT in the hippocampus of SERT−/− mice might be due, in part, to decreased serotonergic axonal innervation, in addition to reflecting the effects of the absence of recycled 5-HT.

Reduced 5-HT axon numbers observed at 3–4 months of age in the hippocampus of SERT−/− versus SERT+/+ mice might result from elevated extracellular serotonin levels present during early developmental periods. However, direct assessment of extracellular 5-HT in mice during prenatal or early postnatal periods is not yet technically feasible. Nevertheless, findings from other studies lend support to the hypothesis that increased 5-HT during critical periods alters the development of the serotonin system and its postsynaptic targets. For example, mice administered a monoamine oxidase-A (MAO-A) inhibitor from embryonic day 15 to postnatal day 7 or mice lacking the MAO-A gene altogether show abnormal thalamocortical barrel field formation in the somatosensory cortex (Cases *et al.*, 1996; Rebsam *et al.*, 2002; Vitalis *et al.*, 1998). High 5-HT levels appear to be causal, since early

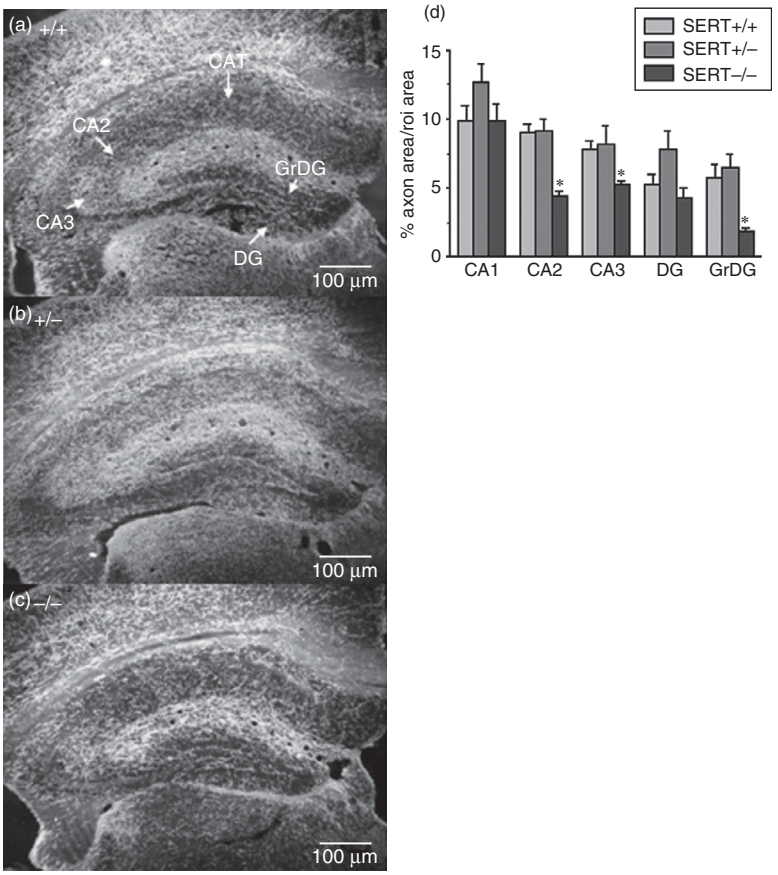

Figure 1.3 Representative darkfield photomicrographs and quantification of 5-HT axons in the hippocampus of 3–4-month-old SERT+/+, SERT+/− and SERT−/− mice visualized by immunocytochemistry and measured by digitized axon analysis. Serotonin axon innervation is significantly decreased in the CA2 and CA3 subregions and the granular layer of dentate gyrus (GrDG) of the hippocampus in SERT−/− mice (c) compared to SERT+/+ mice (a,d). In d, the data are reported as mean percent axon area/region of interest area ± SEM. DG, dentate gyrus. Probabilities are identified as $^{*}p<0.05$, $^{*}p<0.01$ and $^{***}p<0.001$ for differences from SERT+/+ mice.

intervention with the tryptophan hydroxylase inhibitor *p*-chlorophenylalanine (*p*CPA) to block 5-HT synthesis in MAO-A-deficient mice restores normal barrel formation (Cases *et al.*, 1996). Furthermore, mice lacking genes for both MAO-A and 5-HT1B receptors possess normal barrel patterns, suggesting that excess stimulation of 5-HT1B receptors by high extracellular 5-HT levels underlies the disruption of barrel formation

(Salichon *et al.*, 2001). Mice lacking SERT demonstrate similarly disrupted barrel patterns (Persico *et al.*, 2001; Salichon *et al.*, 2001). Additionally, SERT gene deletion has been reported to be associated with reduced numbers of serotonin immunopositive cell bodies in the dorsal raphe (Lira *et al.*, 2003).

In both the Lira study and the data presented above, antiserotonin antibodies were used to visualize serotonergic neurons and their projections. The possibility exists that decreases in stained structures detected might reflect decreased tissue 5-HT levels (Bengel *et al.*, 1998; Kim *et al.*, 2005). However, 5-HT-immunopositive staining is associated with amplification occurring at the levels of secondary antibody binding and conversion of substrate to product by horseradish peroxidase-conjugated secondary antibodies (Mamounas and Molliver, 1988; Mamounas *et al.*, 1991). All immuno-positive structures with staining above a minimal threshold are detected and treated similarly in the digital imaging and quantification routines, regardless of the staining intensity. In this way, a reduced immunocytochemical signal is not necessarily expected when neurotransmitter levels are decreased. A more detailed discussion of this method, including additional information on interpreting immunocytochemical data, can be found in Luellen *et al.* (2006).

Similar to the other aspects of presynaptic serotonergic system structure and function discussed, there is disagreement in the literature as to the effects of chronic pharmacological inhibition of SERT on serotonergic innervation, although much less work has been done in this area. Recently, Williams and coworkers reported a threefold *reduction* in the density of 5-HT fibers in the inferior colliculus that was accompanied by modest reductions in 5-HT axon numbers in frontal and visual cortices in adult male mice chronically treated with imipramine or citalopram for 4 weeks (Williams *et al.*, 2005). No differences in 5-HT axon densities were observed in the pons, superior colliculus, motor cortex, or cerebellum in antidepressant-treated versus vehicle-treated mice. Quantitative analysis of the hippocampus was not carried out in this study.

By contrast, Zhou *et al.* demonstrated an *increase* in serotonergic innervation of the fronto-parietal neocortex (especially layers IV and V), primary olfactory (piriform) cortex, and subcortical limbic structures, including the ventral pallidum and nucleus accumbens shell, in 4–6-month-old male rats given either fluoxetine or tianeptine but not desipramine for 4 weeks (Zhou *et al.*, 2006). Effects in the hippocampus were not reported in this study either. No changes in tryptophan hydroxylase

type-2 mRNA (TPH-2, the rate-limiting enzyme for 5-HT biosynthesis) or SERT mRNA levels were found in the raphe nuclei in fluoxetine-treated rats as determined by RT-PCR. The authors suggest that the increase in 5-HT- or SERT-immunopositive axons was not due to increased synthesis or uptake of 5-HT. Anterograde tracing of raphe axons with biotinylated dextran amine (BDA) revealed increased axonal branching in the piriform cortex in fluoxetine-treated animals (Zhou *et al.*, 2006). It is interesting to note that the atypical antidepressant tianeptine, which has been described as a serotonin reuptake enhancer, had similar effects to the SSRI fluoxetine in this study. These authors speculated that in both cases serotonin-specific effects on BDNF to promote sprouting in forebrain regions (Mamounas *et al.*, 2000) might be at work. In a separate study, it was postulated that upregulation of SERT expression in the frontal cortex, determined by [^{3}H]paroxetine binding in male rats receiving chronic fluoxetine treatments, might reflect increased 5-HT axonal growth and synaptogenesis in this region, possibly caused by 5-HT itself and/or the neurotrophic peptide, S100β (Wegerer *et al.*, 1999). The latter two arguments support the theory that increased plasticity is a key facet of antidepressant efficacy (Castren, 2004).

One difference between the study by Williams *et al.* and those carried out by Zhou *et al.* and Wegerer *et al.* is that mice were investigated in the former while rats were studied in the latter (Wegerer *et al.*, 1999; Williams *et al.*, 2005; Zhou *et al.*, 2006). It is possible that responses of mice versus rats to chronic antidepressant treatment might be different and contribute to the opposing changes in 5-HT innervation patterns observed in these studies. All of these studies utilized male animals in their experiments. It will be of interest to investigate the effects of chronic antidepressant administration on 5-HT innervation in female mice and rats in future studies to ascertain potential gender-related effects.

An important difference between studies on chronic antidepressant treatment compared to those focused on constitutive decreases in SERT expression is that reduced 5-HT reuptake occurs during critical prenatal and/or postnatal developmental periods in the latter. Similar to SERT-deficient mice and rats, increases in depressive behavior and altered reactivity to novel environments have been reported to occur following perinatal exposure of rats (Andersen *et al.*, 2002; Hansen *et al.*, 1997; Hilakivi and Hilakivi, 1987; Mirmiran *et al.*, 1981; Velazquez-Moctezuma and Diaz Ruiz, 1992; Vogel *et al.*, 1990), or postnatal exposure of mice to antidepressants (Ansorge *et al.*, 2004; Lisboa *et al.*, 2007;

Popa *et al.*, 2008). In mice, this effect does not appear to be associated with chronic inhibition of norepinephrine (Ansorge *et al.*, 2008) during the early postnatal period. Changes in mice treated during adulthood with fluoxetine (Ansorge *et al.*, 2008) or escitalopram (Popa *et al.*, 2008) do not produce similar effects and chronic administration of fluoxetine during adulthood reversed heightened depressive-like behaviors produced by postnatal treatment with escitalopram in mice (Popa *et al.*, 2008). Thus, inhibition of 5-HT uptake during a well-defined developmental window appears to recapitulate some, although not all, of the aspects of the phenotype associated with constitutive SERT deletion. Additionally, expression of 5-HT1A receptors during perinatal development is necessary to establish normal anxiety-like behavior in adult mice (Gross *et al.*, 2002). Together, these studies and others imply that alterations in serotonin neurotransmission during key periods of development induce life-long changes in neurochemistry, neuroanatomy and behavior that may be different from those produced by chronic treatment in adulthood with antidepressants.

v. conclusions

Although constitutive loss of one functional copy of the SERT gene leads to a 50% reduction in 5-HT reuptake in SERT+/− mice (Perez and Andrews, 2005; Perez *et al.*, 2006) that is accompanied by a 1–2-fold increase in extracellular 5-HT levels (Mathews *et al.*, 2004), the presynaptic serotonergic system appears capable of maintaining normal intracellular 5-HT levels (Bengel *et al.*, 1998; Numis *et al.*, 2004) and hippocampal serotonergic innervation. By contrast, complete constitutive loss of SERT is associated with dramatic changes in presynaptic homeostasis as reflected by increases in extracellular 5-HT on the order of 6–10-fold when corrected for in vivo recovery (Mathews *et al.*, 2004), and decreases in intracellular 5-HT levels ranging from 40 to 80% (Bengel *et al.*, 1998; Fabre *et al.*, 2000; Kim *et al.*, 2005). The latter may result from both a loss of recaptured 5-HT that is normally taken back up by SERT, as well as a decrease in the number of serotonergic axons, although additional work will need to be done to determine whether changes in innervation occur in other regions of the forebrain in SERT−/− mice. In SERT−/− mice, an increase in in vivo 5-HT synthesis rates that are not due to changes in TPH-2 levels also occurs (Kim *et al.*, 2005). Reuptake of 5-HT by alternate transporters under conditions of complete constitutive loss of SERT may also take place in some brain regions (Schmitt *et al.*, 2003; Zhou *et al.*, 2002), which, in addition to increased synthesis, may partly

contribute to the homeostatic maintenance of tissue 5-HT levels in SERT−/− mice.

In humans, Lesch and Murphy have identified a 43-base pair insertion/deletion (indel) polymorphism (originally thought to be a 44 bp indel) in the promoter region of the human serotonin transporter gene (Heils *et al.*, 1996; Wendland *et al.*, 2006). Men and women expressing one or two copies of the short form of the 5-HTTLPR gene variant score higher on measures of anxiety-related personality traits (Greenberg *et al.*, 2000; Lesch *et al.*, 1996). This important discovery has spawned numerous studies aimed at replicating and extending these results. Two recent meta-analyses found consistent associations between the 5-HTTLPR short allele and neuroticism, a trait related to anxiety, hostility, and depression, on the NEO Personality Inventory Scales (Schinka *et al.*, 2004; Sen *et al.*, 2004).

The 5-HTTLPR is postulated to produce alterations in anxiety-related traits by driving allele-specific SERT promoter activity, giving rise to a 40% variation in SERT mRNA levels in heterologous expression systems (Heils *et al.*, 1996; Lesch *et al.*, 1996). Furthermore, 40% decreases in SERT protein expression in postmortem human brain and [^{3}H]5-HT uptake in human lymphoblasts and platelets are reported to be associated with the short allele (Greenberg *et al.*, 1999; Lesch *et al.*, 1996; Little *et al.*, 1998). However, recent studies on human SERT binding by PET in vivo and in postmortem frontal cortex, as well as on SERT mRNA levels in human raphe tissue, are not in agreement with earlier findings (Lim *et al.*, 2006; Mann *et al.*, 2000; Parsey *et al.*, 2006a). In each of these cases, the authors hypothesized that the effects of the 5-HTTLPR to reduce SERT expression might be limited to developmental periods. The results of the animal studies described above involving perinatal administration of serotonin reuptake inhibiting antidepressants further suggest that genetically driven decreases in SERT expression, whether constitutive or limited to key periods of development, might have their greatest influence during early life in humans and animals. In any case, many studies in humans suggest that reduced SERT expression and function, whether present throughout life or limited to development, contribute to increases in adult anxiety-related behavior and susceptibility to major depressive disorder observed (Caspi *et al.*, 2003; Grabe *et al.*, 2005; Neumeister *et al.*, 2002, 2006; Parsey *et al.*, 2006b; Pezawas *et al.*, 2005; Zalsman *et al.*, 2006).

Drawing conclusions from the extensive literature on the effects of chronic antidepressant treatment on adaptive responses in the presynaptic serotonergic system is more difficult. There appears to be

some consensus across studies that long-term administration of antidepressants, and in particular SSRIs, leads to a decrease in SERT. However, it is not yet clear whether this involves a downregulation of protein expression and/or a redistribution of SERT from the plasma membrane. The majority of studies also point to decreased tissue 5-HT levels occurring in response to chronic antidepressant treatment. By contrast, we examined approximately 50 microdialysis papers utilizing various regimens of antidepressant administration and discovered that almost half of these reported no change in dialysate 5-HT levels, as opposed to the often hypothesized increases in dialysate 5-HT reported in the other half of the studies. We were unable to rectify these differences by considering brain region, drugs or drug class, dose, route of administration, or washout period. In fact, even when zero net flux microdialysis was used in mice to correct for in vivo probe recovery, a single study on antidepressant treatment concluded that increases in extracellular 5-HT were not present in hippocampus or frontal cortex following continuous delivery of therapeutic levels of paroxetine (Gardier *et al.*, 2003). These results contrast with our findings using zero net flux in SERT-deficient mice where increases in extracellular 5-HT were detected in SERT+/− mice, as well as SERT−/− mice (Mathews *et al.*, 2004). Finally, the findings of two immunocytochemical studies that reported on changes in forebrain axon densities following chronic antidepressant treatment were in opposition to each other (Williams *et al.*, 2005; Zhou *et al.*, 2006). Neither study specifically examined the hippocampus so comparisons cannot be made to the data we report here on decreased axonal innervation occurring in some but not all subregions of hippocampus in mice lacking SERT.

There is a wide range of drug administration protocols that has been utilized in the studies discussed above, and this likely contributes to some of the conflicting findings. It will be important for future studies to consider closely the methods of drug delivery employed. There is some evidence that administration by osmotic minipump to achieve continuous delivery in rodents leads to more consistent results. This was particularly evident in the studies on the effects of chronic antidepressant treatment on SERT levels. Serum or plasma drug levels resulting from different administration paradigms and drug doses will need to be investigated further to ensure that human therapeutic levels are reached, as demonstrated by the work of Hirano and coworkers (2005), among others. However, the ultimate test of drug efficacy across species will lie in demonstrating that reuptake is effectively blocked for a period of weeks in experimental animals.

These considerations should be extended to studies aimed at investigating the effects of antidepressants during critical developmental periods. Here, much additional work needs to be done to investigate adaptive responses in the presynaptic serotonergic system following disruption of SERT function in pre- and early postnatal periods, particularly regarding the possible persistence of these responses into adulthood. Elucidating these types of changes will help us to understand the mechanisms underlying lasting alterations in exploratory behavior and a depressive-like endophenotype (Andersen *et al.*, 2002; Ansorge *et al.*, 2004, 2008; Hansen *et al.*, 1997; Hilakivi and Hilakivi, 1987; Mirmiran *et al.*, 1981; Popa *et al.*, 2008; Velazquez-Moctezuma and Diaz Ruiz, 1992; Vogel *et al.*, 1990). These, as well as the many other factors enumerated above, point to the need for the development of conditional SERT-deficient mice. This type of model, similar to what has been done for 5-HT1A receptors (Gross *et al.*, 2002), will allow better controlled investigations of the powerful adaptive forces evoked by long-term loss of serotonin reuptake, as well as the critical temporal windows modulating its pleotropic effects.

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