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NATURAL AND ENGINEERED CODING VARIATION IN ANTIDEPRESSANT-SENSITIVE SEROTONIN TRANSPORTERS

R. YE[a] AND R. D. BLAKELY[a,b,c,d]*

[a]*Department of Pharmacology, Vanderbilt University School of Medicine, Nashville, TN 37232-8548, USA*

[b]*Department of Psychiatry, Vanderbilt University School of Medicine, Nashville, TN 37232-8548, USA*

[c]*Center for Molecular Neuroscience, Vanderbilt University School of Medicine, Nashville, TN 37232-8548, USA*

[d]*Silvio O. Conte Center for Neuroscience Research, Vanderbilt University School of Medicine, Nashville, TN 37232-8548, USA*

Abstract—The presynaptic serotonin (5-HT) transporter (SERT) is a key regulator of 5-HT signaling and is a major target for antidepressant medications and psychostimulants. In recent years, studies of natural and engineered genetic variation in SERT have provided new opportunities to understand structural dimensions of drug interactions and regulation of the transporter, to explore 5-HT contributions to antidepressant action, and to assess the impact of SERT-mediated 5-HT contributions to neuropsychiatric disorders. Here we review three examples from our recent studies where genetic changes in SERT, identified or engineered, have led to new models, findings, and theories that cast light on new dimensions of 5-HT action in the CNS and periphery. First, we review our work to identify specific residues through which SERT recognizes antagonists, and the conversion of this knowledge to the creation of mice lacking high-affinity antidepressant and cocaine sensitivity. Second, we discuss our studies of functional coding variation in SERT that exists in commonly used strains of inbred mice, and how this variation is beginning to reveal novel 5-HT-associated phenotypes. Third, we review our identification and functional characterization of multiple, hyperactive SERT coding variants in subjects with autism. Each of these activities has driven the development of new model systems that can be further exploited to understand the contribution of 5-HT signaling to risk for neuropsychiatric disorders and their treatment.

Key words: serotonin, antidepressant, transporter, cocaine, autism, p38 mitogen-activated protein kinase.

Contents

*Correspondence to: R. D. Blakely, Suite 7140 MRBIII, Vanderbilt University School of Medicine, 465 21st Avenue South, Nashville, TN 37232-8548, USA. Tel: +1-615-936-3705; fax: +1-615-936-3040.
E-mail address: randy.blakely@vanderbilt.edu (R. D. Blakely).
Abbreviations: SERT, serotonin transporter; SSRI, 5-HT selective reuptake inhibitors.

Presynaptic, 5-hydroxytryptamine (5-HT, serotonin) transporters (SERTs, *SLC6A4*) mediate the clearance and reuptake of 5-HT during synaptic transmission (Ramamoorthy et al., 1993a). SERTs are highly expressed by midbrain raphe nuclei serotonergic neurons, beginning at the earliest stages of 5-HT neuron differentiation (Schroeter and Blakely, 1996; Hansson et al., 1998; Hendricks et al., 2003; Wylie et al., 2010). Although restricted in expression to 5-HT neurons in the adult, SERTs are expressed by nonserotonergic neurons during development (Hansson et al., 1998, 1999; Gaspar et al., 2003). Along with expression by the placenta (Ramamoorthy et al., 1993b), these latter sites are thought to coordinate the availability of 5-HT to act in the modulation of CNS axon guidance and synapse formation (Bonnin et al., 2007). Besides the placenta, SERTs are expressed in platelets (Mercado and Kilic, 2010), lymphocytes (Barkan et al., 2004), pancreatic beta cells (Smith et al., 1999), pulmonary epithelium (Bhat and Block, 1990), bone (Bliziotes et al., 2001), and the gut mucosa (Chen et al., 2001). Whereas the role of SERT in restricting access of targets to 5-HT and in acquiring 5-HT for release has been well-studied, the acquisition of 5-HT for transglutaminase II-catalyzed covalent attachment to small GTP-binding proteins that can modulate the fusion of secretory granules (Walther et al., 2003), and 5-HT generated oxidative stress and stress-activated MAPK/Rho kinases have emerged recently as yet other mechanisms by which SERT influences physiology (Guilluy et al., 2009; Liu et al., 2011). Together, these (and likely other) actions of SERT reveal a broader range of physiological actions of the transporter in the brain and periphery than previously understood and that will require new approaches and models to clarify.

Inhibitors of SERT, including the 5-HT selective reuptake inhibitors (SSRIs), are well known for their use in the treatment of anxiety disorders, depression, and obsessive-compulsive disorders. Naturally, therefore, polymorphisms in the SERT gene have been extensively studied in humans as potential risk determinants of neuropsychiatric disorders (for detailed reviews, see Lesch et al., 1996; Hahn and Blakely, 2007). Early studies identified two common polymorphisms in the promoter region (5-HTTLPR)

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and intron 2 (VNTR-2) of *SLC6A4* (Lesch et al., 1996; Fiskerstrand et al., 1999). The 5-HTTLPR, a polymorphic, repeat structure 5′ of the sites of initiation for SERT RNA transcription. Initial studies indicated an association of the 5-HTTLPR with anxiety traits in nonclinical subjects and, more recently, in risk for depression and suicide, particularly in the context of early adverse life events (Caspi et al., 2003; Wankerl et al., 2010). These studies, however, have not met with consistent replication, though most recent analyses across larger number of studies where consistency of studies can be maintained, point to significant effects when specific components of mood disorders are assessed (Blakely and Veenstra-Vanderweele, 2011).

As common, genetic variation such as the 5-HTTLPR is often found in noncoding or complex regulatory regions of genes, it can be difficult to establish mechanistic contributions to disease risk. On the other hand, *SLC6A4* coding variation is rare, with the most common variation (Gly56Ala in the SERT N-terminus) exhibiting an allele frequency generally under 1% (Glatt et al., 2001; Sutcliffe et al., 2005). Though the low frequency of these structural changes in SERT protein might appear seemingly irrelevant to population disease risk, they can be quite functionally penetrant (Prasad et al., 2009), and are easier to study *in vitro* since SERT activity and regulation can be reconstituted following heterologous expression. Such efforts can permit the identification of broader mechanisms whose compromised control of SERT expression, activity, and regulation could phenocopy SERT-influenced disorders and even identify new targets within which more common gene variation can contribute more broadly to disease risk (Veenstra-Vanderweele and Blakely, in press). Indeed, in several cases, familial segregation of rare SERT variants has provided strong support for a contribution of disrupted 5-HT signaling neuropsychiatric disorders, including OCD (Ozaki et al., 2003) and autism (Sutcliffe et al., 2005; Prasad et al., 2009). These studies draw attention to a network of SERT-regulatory signaling pathways that may bear additional disease-associated gene variation, or be modified by epigenetic influences, such as early-life adverse events. Although these alterations can institute limits to the behavioral flexibility needed to deal with everyday life, their origins in perturbed 5-HT signaling suggest opportunities for pharmacological treatments that, properly targeted, can provide relief from many disabling symptoms.

The translocation of 5-HT across the plasma membrane by SERT is a multi-step process that involves the binding of neurotransmitter along with Na^+/Cl^-, followed by protein conformational changes, the intracellular release of substrates, and the binding of K^+ to drive return of the unloaded carrier for subsequent rounds of transport. Together, these steps energize the 5-HT transport mechanism and provide multiple steps for regulation of SERT activity. But exactly where critical events, such as 5-HT binding, internal occlusion, and membrane transit, occur in SERT have only recently begun to emerge. Since the cloning of SERT cDNAs two decades ago (Ramamoorthy et al., 1993a), many mutation studies have been conducted with the hope of pinpointing key features of substrate and antagonist binding, as well as determinants of transporter regulation. The high-resolution crystal structure of a bacterial SLC6 transporter, $LeuT_{Aa}$ (Yamashita et al., 2005; Singh et al., 2008), provided a critical framework on which the effects of such could be appreciated in molecular terms. Unfortunately $LeuT_{Aa}$ lacks key cytoplasmic sequences (most of the N- and C-termini and cytoplasmic loops are either unordered or absent) that have been implicated in transporter regulation. As we will discuss later in the text, the $LeuT_{Aa}$ crystal structure has validated our hypotheses concerning the location of 5-HT and high-affinity antagonist-binding sites in SERT and encouraged further exploration of their manipulation *in vivo*.

Structural determinants of SERT regulation remain to be fully elucidated, though a combination of engineered and natural gene variation has provided remarkable opportunities to elucidate opportunities for drug development as well as to elucidate normal regulatory networks where disruption can support neuropsychiatric diseases associated with compromised 5-HT signaling. Neuropsychiatric diseases are polygenic in nature and feature many opportunities for environmental triggers or modulation. Thus, though the connections between SERT dysfunction and disorders such as depression and autism continue to be explored, deciphering the exact roles of SERT requires a more nuanced understanding of (1) the transporter's interactions with other gene products, (2) a careful dissection of where among the many sites of SERT expression such interactions are relevant, and (3) whether these interactions are most critical during early life or in the adult (or both). In this context, the discussions of our efforts to examine the impact of natural and engineered genetic variation in this review should be seen not as a comprehensive review of the literature, but more as an outline of possible new entry points and tools that can assist us in understanding the complex roles of SERT and 5-HT in human behavior and drug action.

IDENTIFICATION OF KEY SITES FOR SSRI INTERACTIONS AT SERT: Tyr95 AND Ile172

Before the structural insights afforded by the $LeuT_{Aa}$ structure, we developed a new approach to probe SERT structure, species-scanning mutagenesis (Barker et al., 1998; Adkins et al., 2001; Henry et al., 2006b), whereby amino acid sequences are sequentially interconverted between SERT species variants, to search for sites of high-affinity interaction of 5-HT and SSRIs. Key to this effort is the fact that many SSRIs, including citalopram and fluoxetine, as well as 5-HT analogs (but not 5-HT itself) display substantial differences in potency for inhibition of 5-HT uptake at human and *Drosophila melanogaster* SERT. By switching amino acid residues in human SERT to their fly counterparts (and vice versa) in regions of the transporter implicated by chimera studies in 5-HT or SSRI recognition, we identified Tyr95 and Ile172 in human SERT as two major structural determinants for binding of many antidepressant medications, as well as cocaine (Barker et al., 1998; Henry et al., 2006b). With the elucidation of the $LeuT_{Aa}$ structure, the location of Tyr95 and Ile172 in