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Serotonin Transporter-Independent Actions of the Antidepressant Vortioxetine As Revealed Using the SERT Met172 Mouse

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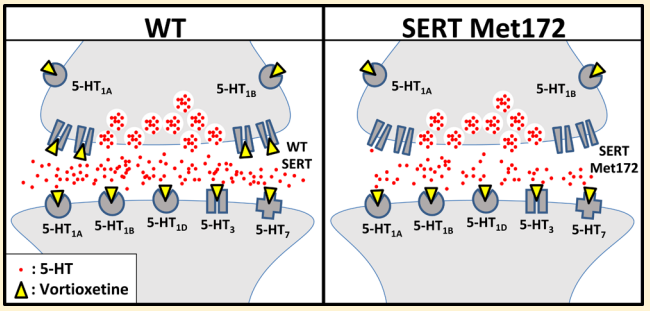
ABSTRACT: Selective serotonin (5-HT, SERT) reuptake inhibitors (SSRIs) are the most commonly prescribed treatments for depression. However, they have delayed efficacy and can induce side-effects that can encourage discontinuation. Recently, agents have been developed, including vortioxetine (Trintellix), that augment SERT blockade with interactions at other targets. At therapeutic doses, vortioxetine interacts with SERT as well as 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_3$, and 5-HT$_7$ receptors. We assessed the SERT-dependency of vortioxetine action using the SERT Met172 mouse model, which disrupts high-affinity interactions of many antidepressants with the transporter. We demonstrate that the SERT Met172 substitution induces an ∼19-fold loss in vortioxetine potency for SERT inhibition in midbrain synaptosomes. Moreover, in these mice, we observed reduced SERT occupancy, a diminished ability to prolong 5-HT clearance, and a reduced capacity to elevate extracellular 5-HT. Despite reduced interactions with SERT, vortioxetine maintained its ability to enhance mobility in tail suspension and forced swim tests, reduce consumption latency in the novelty induced hypophagia test, and promoted proliferation and survival of subgranular zone hippocampal stem cells. Our findings suggest that the antidepressant actions of vortioxetine may be SERT-independent, and encourage consideration of agents that mimic one or more actions of the drug in the development of improved depression treatments.



KEYWORDS: *Serotonin, vortioxetine, mouse, SERT Met172, antidepressant*

INTRODUCTION

Depression is the most prevalent mental illness, with incidence rates approaching 7%[1] and a lifetime incidence at 17%,[2] and is a leading contributor to global disease burden.[3] The most common antidepressant medications are the selective serotonin (5-HT) reuptake inhibitors (SSRI), which act to block the ability of the 5-HT transporter (SERT) to clear 5-HT following synaptic release, elevating extracellular levels of the neurotransmitter and 5-HT signaling. Despite their widespread use, SSRIs have limited utility due to high nonresponder rates, side effects and delayed onset of efficacy.[4] Although the evidence for 5-HT as a modulator in brain circuits supporting mood and anxiety is substantial,[5,6] efforts to improve antidepressant efficacy have resulted in novel small molecules that retain SERT inhibition but add interactions with one or more other targets. One recently approved medication is vortioxetine (Trintellix), an agent that among other targets, combines high-affinity SERT inhibition with agonism of 5-HT$_{1A}$, receptors, partial agonism of 5-HT$_{1B}$ receptors, and antagonism of 5-HT$_3$ and 5-HT$_7$ receptors.[7] As the complexity of target engagement grows, it becomes more difficult to attribute drug action to any one target. Indeed, many 5-HT receptor-directed ligands demonstrate antidepressant-like effects in animal behavioral studies.[8,9] For example, antidepressant-like effects can be produced by activation of 5-HT$_{1A}$,[10] 5-HT$_{1B}$,[11] 5-HT$_{2C}$,[12] and 5-HT$_4$[13] receptors or via antagonism of 5-HT$_{2A}$,[14] 5-HT$_3$,[15] and 5-HT$_7$[16] receptors. We therefore reasoned that SERT inhibition by vortioxetine might contribute little to the molecule's antidepressant efficacy.

One strategy to evaluate the SERT dependence of vortioxetine action is to compare the actions of the antidepressant in wild-type and SERT knockout mice/rats.[17,18] However, SERT knockout mice display a significant number of compensatory alterations in biochemistry, circuitry, physiology and pharmacology including reductions in CNS tissue 5-HT levels[19] and 5-HT neuron density,[20] altered dorsal 5-HT neuron firing rates,[20] and changes in the density/sensitivity of multiple 5-HT receptors.[21−24] Although SERT KO rats have been more recently developed,[17] existing studies also indicate significant compensatory alterations that confound interpretations of the role of SERT in antidepressant action. These considerations encouraged our search for a strategy

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whereby antidepressant recognition by SERT would be reduced while preserving normal SERT protein expression, surface trafficking, 5-HT recognition and 5-HT transport capacity. Through a comparison of the species-dependent potencies of antidepressants at SERT, we tracked a major determinant of the comparatively low potency of multiple SSRIs at *Drosophila melanogaster* SERT to a single residue, Met172, present in SERT transmembrane domain 3, where in humans an Ile residue is found.[25] Subsequent studies revealed that a human or mouse SERT with the Met172 exhibits 10−1000 fold changes in 5-HT uptake inhibitory potency for most antidepressants without a change in SERT surface expression or 5-HT transport function. Importantly, these properties were retained in the SERT Met172 knock-in mouse, providing a tool to assess the SERT (and thereby the 5-HT) dependence of SSRI action.[25−27] Recently, we used the model to demonstrate that multiple preclinical measures of fluoxetine and citalopram action, including both acute and chronic actions, are eliminated in the SERT Met172 model,[27] whereas a developmental action of citalopram is SERT-independent.[28]

In the current report, we implement the SERT Met172 model to assess the SERT contributions to the actions of vortioxetine. Our studies reveal a strikingly distinct impact of the SERT Met172 mutation than encountered with SSRIs to date,[27] where despite a loss of potency for SERT blockade, the activity of vortioxetine in multiple preclinical measures of antidepressant action was retained. These findings underscore the unique pharmacological profile of vortioxetine and suggest that further refinement of this molecule could lead to agents that retain antidepressant efficacy in the absence of SERT inhibition altogether.

RESULTS AND DISCUSSION

Vortioxetine Demonstrates Reduced Interactions with SERT Met172. In order to consider the SERT Met172 substitution as a tool to evaluate SERT contributions to vortioxetine action, we first analyzed whether vortioxetine is sensitive to the SERT Met172 substitution utilizing transfected HEK293T cells. In previous analyses, we determined that the Ile172Met substitution is inconsequential to SERT protein expression or transport function.[25−27] In transport inhibition studies, we observed a significant, ∼19-fold reduction in vortioxetine inhibitory potency for 5-HT uptake inhibition comparing SERT Met172 (K_I = 411.0 ± 1.1 nM) versus WT SERT transfected cells (K_I = 22.0 ± 0.1 nM) (Figure 1A). Consistent with these studies, 5-HT uptake sensitivity to vortioxetine was reduced in synaptosomes derived from SERT Met172 mice (K_I = 262.0 ± 3.0 nM) compared to 5-HT uptake measured from WT SERT synaptosomes (17.1 ± 0.2 nM) (Figure 1B), corresponding to an ∼19-fold reduction.

To assess SERT binding interactions for vortioxetine *in vivo*, and define a subsaturating dosage in WT animals that would permit discrimination of SERT Met172 effects, we pursued ex vivo monitoring of SERT occupancy after i.p. vortioxetine injections. In these studies, we probed brain sections from both WT and SERT Met172 mice for the density of available SERT binding sites using radiolabeled paroxetine, which is SERT Met172-insensitive.[25] These analyses revealed that both 5 and 10 mg/kg vortioxetine resulted in significant SERT occupancy in WT animals, nearing or exceeding the SERT occupancy required for acute antidepressant efficacy of traditional pure SSRIs (∼80%).[29] At both doses, SERT Met172 mice displayed significant reductions in vortioxetine SERT occupancy compared to WT mice (Figure 1C). While SERT Met172 mice display significant SERT occupancy at both doses of vortioxetine, these levels do not approach the SERT occupancy reported to be required for SSRI efficacy.[29] As we could be certain to obtain sufficient SERT occupancy in WT animals for SSRI-like effects, we focused further efforts on the 10 mg/kg dose.

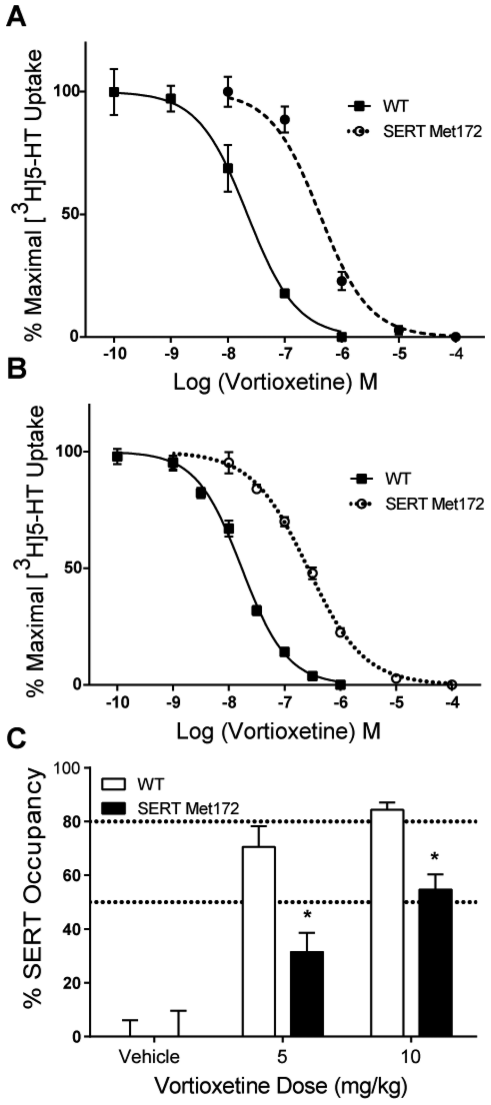

Figure 1. SERT Met172 disrupts vortioxetine binding. In vitro cellular uptake (A). Transfected HEK293T cells with hSERT or hSERT-Met172 display altered pharmacological sensitivity to vortioxetine. WT 5-HT uptake was inhibited by vortioxetine with a K_I = 2.20 ± 0.01 × 10^{-8} M whereas SERT Met172 was inhibited by vortioxetine with a K_I = 4.11 ± 0.11 × 10^{-7} M. $P < 0.05$, Student's t test, n = 4 per genotype and condition. Synaptosomal uptake (B). The SERT Met172 substitution imposes significantly reduced sensitivity to vortioxetine compared to WT SERT. Vortioxetine (WT K_I = 1.71 ± 0.02 × 10^{-8} M; SERT Met172 K_I = 2.62 ± 0.03 × 10^{-7} M; $P < 0.05$, Student's t test, n = 4 per genotype and condition. Ex vivo SERT occupancy (C). SERT occupancy ex vivo following acute administration of vortioxetine (5 and 10 mg/kg i.p.). Mice were pretreated with vortioxetine for 1 h prior to sacrifice. SERT Met172 mice display significant reductions in SERT occupancy compared to WT animals following vortioxetine administration. Data are presented as mean ± SEM (n = 4 per genotype and condition). *$P < 0.05$, two-way ANOVA followed by Fisher's protected t post hoc test.

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Vortioxetine at 10 mg/kg i.p. Fails to Delay 5-HT Clearance and Elevate Extracellular 5-HT in Vivo in SERT Met172 Mice. In order to assess whether the reduction in SERT occupancy by vortioxetine arising in SERT Met172 mice is sufficient to impact extracellular 5-HT homeostasis *in vivo*, we utilized two approaches, *in vivo* chronoamperometry and microdialysis. Chronoamperometry provides a measure of real-time 5-HT clearance capacity via carbon fiber oxidation of pulse-applied 5-HT.[30] SERT inhibition using this approach is revealed by a delay in the amount of time required to clear 80% (T_{80}) of applied 5-HT from peak signal amplitiude,[31] an effect lost in the SERT Met172 model.[26] In these studies, we assessed 5-HT clearance rates in the CA3 region of the dorsal hippocampus every 10 min for 1 h. Example raw traces are provided in Figure 2A, and averaged T_{80} data in Figure 2B. Baseline 5-HT clearance rates showed no difference between genotypes (data not shown). Compared to predrug baseline, vortioxetine increased 5-HT T_{80} in WT mice, but not in SERT Met172 mice.

Because *in vivo* chronoamperometry monitors the clearance of locally applied 5-HT, we complemented these studies with an assessment of the impact of SERT Met172 on vortioxetine-induced changes in endogenous levels of extracellular 5-HT utilizing in vivo microdialysis. SSRIs, such as fluoxetine and citalopram, can elevate extracellular levels of 5-HT in microdialysates in WT but not SERT Met172 mice at behaviorally relevant doses.[26] At 10 mg/kg i.p., we found that vortioxetine produced a readily detectible elevation of extracellular 5-HT in the hippocampus of WT mice, whereas little effect was evident at the same dose in SERT Met172 mice (Figure 2C).

Vortioxetine Retains Behavioral Efficacy in the Tail Suspension Test (TST) and Forced Swim Test (FST) in SERT Met172 Mice Following Acute Administration. With a dose of vortioxetine defined that achieves high occupancy and significant functional SERT inhibition in WT but not SERT Met172 mice in vivo, we moved to determine whether vortioxetine would retain actions typical of acute or chronically administered antidepressants. We also assessed the actions of the SSRI paroxetine (20 mg/kg i.p,) in parallel to demonstrate a lack of effect of the Met172 mutation on other components of the antidepressant response pathway as this agent has been previously demonstrated to be insensitive to the SERT Met172 substitution in vitro[25] and in vivo.[26,27] WT animals in the TST (Figure 3A) and FST (Figure 3B) display significantly increased time mobile relative to vehicle condition following acute injections of either vortioxetine or paroxetine. As expected, SERT Met172 mice also displayed significant increase in mobility in response to paroxetine. We found that paroxetine had a larger effect size than vortioxetine in the TST, but not the FST. As increased efficacy of paroxetine relative to vortioxetine in the TST was not genotype-dependent, we attribute this effect to a higher affinity of paroxetine for SERT and a greater dynamic range of the TST versus the FST in our study of SERT Met172 mice. Notably, these mice also show significant increases in mobility following vortioxetine administration. These findings stand in contrast to our prior findings of a loss of efficacy for both citalopram and fluoxetine in the TST and FST in SERT Met172 mice,[27] suggesting that aspects of vortioxetine pharmacodynamics other than SERT inhibition (e.g., 5-HT receptor interactions) can drive its acute antidepressant-like actions. Support for this hypothesis is further evident in an examination of the climbing component

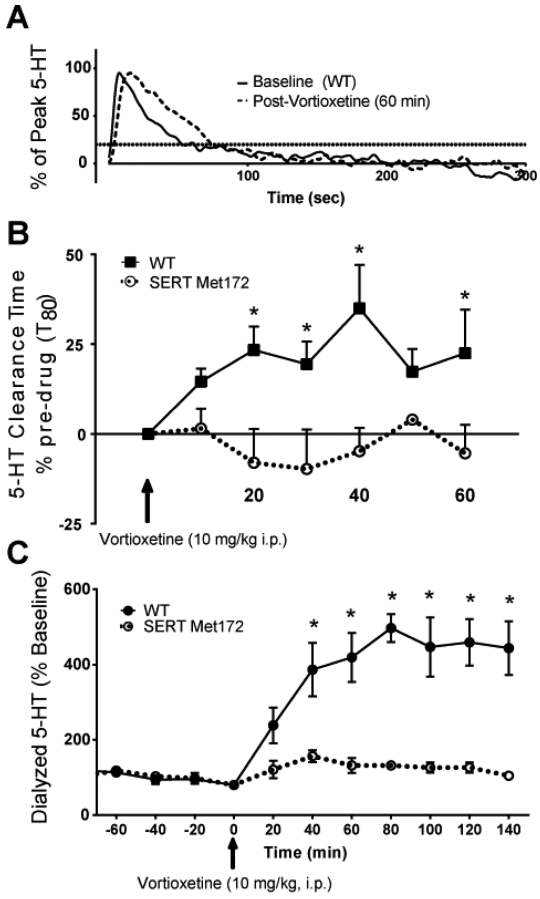

Figure 2. The Met172 allele impairs functional SERT inhibition *in vivo*. Chronoamperometry (A). 5-HT clearance measured in the CA3 region of the hippocampus by *in vivo* chronoamperometry. Example raw chronoamperometry trace for WT mouse 60 min following 10 mg/kg i.p. vortioxetine injection. Dashed line represents a fall by 80% from peak amplitude of the 5-HT response, from which T_{80} is calculated. (B) Following i.p. injection of 10 mg/kg vortioxetine, WT, but not SERT Met172 mice, display delayed 5-HT clearance kinetics, as evidence by an increase in the percent change of T_{80}, assessed every 10 min for 1 h following i.p. drug injection. Microdialysis (C). Measurements of extracellular 5-HT by *in vivo* microdialysis. Time course of the effects of vortioxetine (10 mg/kg, i.p.) on extracellular 5-HT concentrations in the ventral hippocampus in WT and SERT Met172 mice. Upon injection of 10 mg/kg i.p. vortioxetine, WT mice displayed robust increases of extracellular 5-HT, indicative of functional SERT inhibition, whereas SERT Met172 mice did not. For (B) and (C), data were analyzed by a two-way repeated measures ANOVA, where significant main time and genotype effects were found ($P < 0.05$). *$P < 0.05$, from Bonferroni post hoc test at the respective time point post vortioxetine injection as compared to baseline. Data are presented as mean ± SEM (B, $n = 3–5$ per genotype; C, $n = 4$ per genotype).

of FST mobility changes. Vortioxetine has been shown to induce climbing behavior in WT mice in the FST,[32] which is thought to be SERT-independent, due to the agent's ability to enhance NE release in the prefrontal cortex[7] via direct antagonism of 5-HT_3 receptors and a resulting silencing of forebrain cortical GABAergic neurons.[33] Consistent with this model, we observed that vortioxetine increased climbing behavior in both WT and SERT Met172 mice in the FST (Figure 3C), whereas paroxetine was without effect.

SERT Met172 Mice Retain Sensitivity to the Chronic Actions of Vortioxetine in the Novelty Induced

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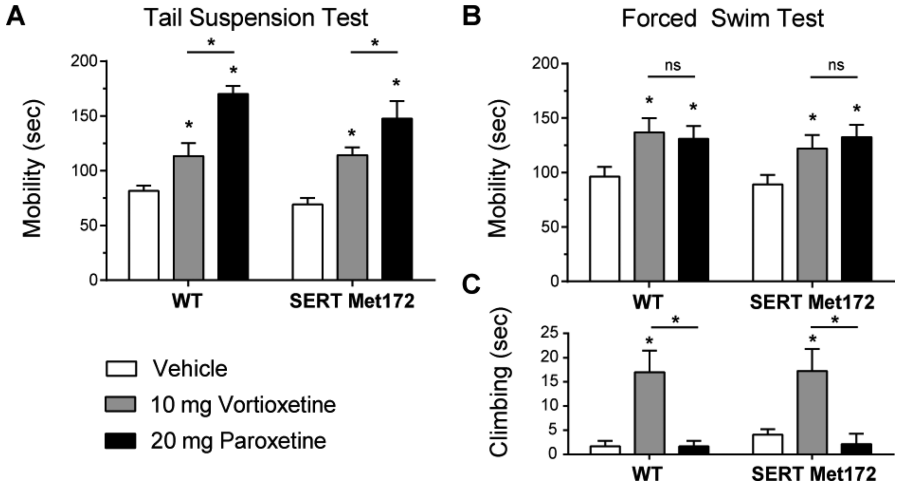

Figure 3. Vortioxetine maintains efficacy in TST and FST in SERT Met172 mice. Actions of vortioxetine in the TST and FST arise independently of SERT inhibition. All tests were performed 60 min after i.p. injection of the concentration of drug listed. Time mobile in a 6 min TST (A). WT and SERT Met172 mice display significant increases in mobility time in response to all drug treatments. Time immobile in a 6 min FST (B). WT and SERT Met172 mice display significant increases in mobility time in response to all drug treatments. WT and SERT Met172 mice display increased climbing behavior following vortioxetine administration (C). Data are presented as mean ± SEM and were analyzed via two-way ANOVA and Holm-Sidak post hoc multiple comparison tests. Asterisk (*) indicates $P < 0.05$ in post hoc tests, either comparing drug to vehicle or comparing between drugs via overhead bar ("ns" indicates nonsignificant, $P > 0.05$). For all measures, $n = 8–12$ per genotype and treatment condition.

Hypophagia (NIH) Test. Although the maintained efficacy of vortioxetine in the TST and FST, two tests with predictive validity of antidepressant action in humans, suggest that the antidepressant actions of vortioxetine may be SERT-independent, these tests do not realistically model the time course of antidepressant action, where multiple weeks of administration are required. The NIH test involves monitoring the latency of animals to approach and consume a known palatable substance in a novel, stressful environment and unlike the TST and FST is only sensitive to chronic, but not acute, antidepressant administration.[34,35] In this paradigm, the latency to approach palatable substance in an aversive environment is reduced by chronic administration of SSRIs.[35] Previously, we demonstrated that both citalopram and fluoxetine lost efficacy in reducing approach latency in SERT Met172 mice, supporting SERT dependence.[27] We administered vortioxetine in the chow at a dose to yield CNS levels equivalent to that found with an acute 10 mg/kg i.p. injection.[36] WT mice displayed the anticipated reduction in latency to consume Vanilla Ensure in a novel, brightly illuminated cage, compared to vehicle administration (Figure 4A). As observed with behavioral tests of acute vortioxetine action, SERT Met172 mice responded like WT animals to both vortioxetine and the active SSRI, paroxetine, displaying equivalent reductions in latency to consume Vanilla Ensure.

Vortioxetine Promotes Hippocampal Neurogenesis in Both WT and SERT Met172 Mice. Another measure responsive to chronic but not acute antidepressant administration is hippocampal neurogenesis, a process that has also been shown to be critical for SSRI efficacy in the NIH test.[34] SSRIs promote both hippocampal stem cell proliferation rate and survival of newly generated hippocampal stem cells,[37] effects that are also observed with the mixed serotonergic antidepressant vortioxetine.[32] Consistent with these findings, we observed that WT mice display increased rates of SGZ hippocampal stem cell proliferation and survival following chronic administration of vortioxetine and paroxetine, assessed by BrdU+ immunohistochemistry. Paralleling the results we obtained in the NIH test, and unlike our prior findings with fluoxetine and citalopram,[27] that SERT Met172 mice retained sensitivity to paroxetine and vortioxetine with respect to hippocampal stem cell proliferation (Figure 4B) and survival (Figure 4C). While the difference between paroxetine and vortioxetine in stem cell proliferation versus survival is interesting, these effects were not genotype dependent, and thus it is possible that they reflect the higher affinity of paroxetine in the case of proliferation and a ceiling effect on both antidepressants for our survival measure.

At clinically relevant doses, vortioxetine possesses high-affinity agonist and antagonist actions at distinct 5-HT receptor subtypes, in addition to SERT inhibition.[32] Specific modulation, either via agonism or antagonism, of these 5-HT receptors in preclinical animal models indicate potential viability as antidepressants.[8,9] SSRIs are believed to promote their effects by modulating 5-HT signaling through enhanced signaling at its receptors, though it is not known which 5-HT receptor subtypes, or ensemble thereof, are responsible for the final antidepressant or untoward effects. To our knowledge, there have been no comprehensive studies performed that investigate the best combination of 5-HT receptor ligands to reproduce the antidepressant effects seen in SSRIs in order to decipher the optimal receptor target, or set of targets, for an antidepressant effect without invoking actions at SERT. Previously, we utilized the SERT Met172 mouse model to establish that a lack of high-affinity SERT inhibition abolishes the actions of two highly prescribed SSRIs, fluoxetine and citalopram.[27] Our studies indicate that although vortioxetine exhibits pharmacological and functional inhibition of SERT at doses that induce antidepressant-like behavioral changes, SERT inhibition is likely dispensable for these effects. As our study design utilized a single, chronic exposure end point, additional studies are needed to determine whether the removal of SERT inhibition can accelerate the antidepressant-like actions of vortioxetine, which could be envisioned if delayed positive responses begin to be initiated once early negative responses have disappeared.

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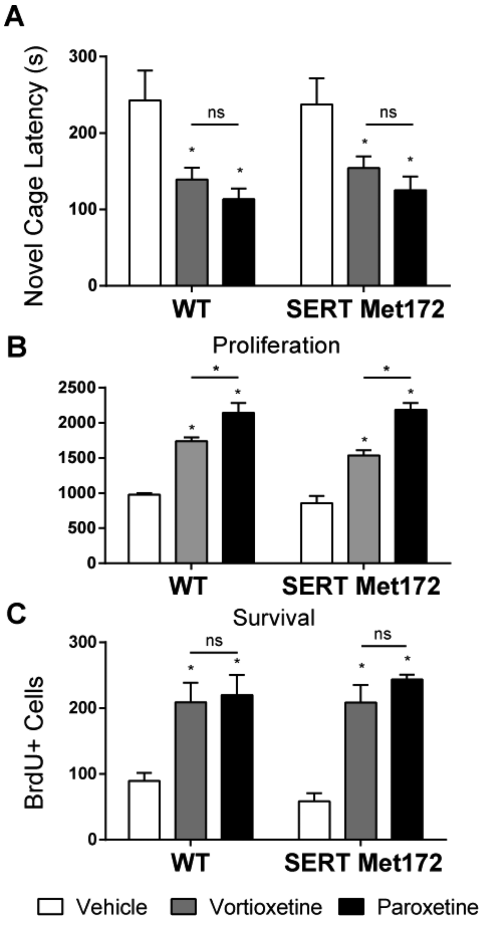

Figure 4. Vortioxetine maintains efficacy with chronic exposure in suppression of NIH and elevation of hippocampal neurogenesis. NIH (A). Chronic vortioxetine remains effective in NIH test in SERT Met172 mice. Latency to consume Vanilla Ensure in novel cage was recorded. WT and SERT Met172 mice display significant reductions in latency following chronic vortioxetine administration. For all measures, n = 16–20 per genotype. Neurogenesis (B,C). Chronic vortioxetine can stimulate hippocampal neurogenesis in SERT Met172 mice. Proliferation (B): Following chronic administration of vortioxetine, WT and SERT Met172 mice display significant increases in stem cell proliferation rate. (n = 4 per genotype and condition). Survival (C): Newly generated stem cells survive significantly more in vortioxetine treated mice than vehicle, an effect preserved in SERT Met172 mice. (n = 4 per genotype and condition). Data are presented as mean ± SEM and were analyzed via two-way ANOVA and Holm-Sidak post hoc multiple comparison tests. Asterisk (*) indicates $P < 0.05$ in post hoc tests, either comparing drug to vehicle or comparing between drugs via overhead bar ("ns" indicates nonsignificant, $P > 0.05$).

An important implication of our studies is that it may be possible to tailor more effective antidepressants by removing the negative consequences of SERT inhibition. Evidence indicates that high occupancy of SERT over several weeks is required for the antidepressant efficacy of SSRIs.[29] A requirement for high occupancy may indicate that a significant excess of SERT activity is present, relative to what is needed to constrain 5-HT actions. Delays in response compared to the rapid occupancy of SERT suggest a complex, time-dependent pattern of signaling plasticities derived from one or more 5-HT receptors. In relation to the latter issue, 5-HT receptor actions resulting from SERT blockade include actions that would be considered both supportive or detrimental to antidepressant actions. For example, excessive 5-HT_{2A} type receptor activation can induce hyperthermia,[38] activation of 5-HT_{2B} produces cardiotoxic events,[39] and 5-HT_3 receptor activation can produce nausea and gastrointestinal discomfort.[40] Although determining the optimum blend of receptor agonism and antagonism is a complex proposition, given the large number of 5-HT receptors, our findings suggest that the spectrum of agonist and antagonistic interactions at 5-HT receptors (activation of 5-HT_{1A}[10] and 5-HT_{1B}[11] receptors and antagonism of 5-HT_3,[15] 5-HT_{1D}, and 5-HT_7[16] receptors) provided by vortioxetine may represent a therapeutically beneficial combination that could be targeted by novel agents lacking SERT inhibition altogether. Such molecules could prove superior to SSRIs by targeting the desired array of 5-HT receptors while better allowing for a dose titration of receptor interactions and avoiding stimulation of other, side-effect generating 5-HT receptors. We cannot rule out that other, nonserotonergic, targets may contribute to vortioxetine's pharmacological effects in the SERT Met172 model. However, a broad in vitro screen of more than 75 targets revealed that, except for the β_1 adrenoceptor (K_I = 46 nM), only weak activities were evident and therefore unlikely to contribute at therapeutic concentrations.[41] Functional studies of β_1 adrenoceptor activity did point to potential pharmacological effects in vivo that should be considered.[41]

We certainly recognize that a major limitation of our study is that our inferences are necessarily gathered from studies with rodents and not humans. Both the TST and FST are used routinely because of their predictive validity, despite acute drug administration. We were thus encouraged that we reached similar conclusions when testing vortioxetine actions in the SERT Met172 model using behavioral and cellular assays with greater face validity in relation to the chronic exposure needed in humans for antidepressant action. We are also aware that our tests of antidepressant action did not involve remediation of behavioral changes attendant to a model of human depression. Studies that examine the impact of the SERT Met172 substitution on the ability of vortioxetine to reverse behavioral or physiological changes observed following chronic, unpredictable stress[42] or chronically elevated corticosterone[43] may be useful in assessing the broader significance of our findings. We also note a recent study indicating that the metabolites of certain antidepressants can retain action at SERT Met172 despite loss of efficacy of the parent molecule.[44] Prior investigations of vortioxetine metabolites concluded that they do not contribute to vortioxetine pharmacological activity in the CNS due to weak activity and low blood brain barrier penetration,[45] suggesting that metabolite contributions to our interpretation of SERT-independent efficacy in SERT Met172 mice is unlikely. Altogether, we believe that our findings justify the possibility that antidepressants with an improved side-effect profile and/or enhanced speed of therapeutic onset could be developed through modifications of vortioxetine to remove SERT inhibition altogether while retaining the actions of the molecule at its other targets, particularly 5-HT receptors.

METHODS

Materials. Unless otherwise noted, reagents used in the current study were derived from Sigma-Aldrich at the highest purity available. Vortioxetine HBr was provided by Lundbeck Research USA (Paramus, NJ); paroxetine HCl was obtained from TCI Chemicals (Portland, OR). For all animal studies, vortioxetine was injected at 5 and 10 mg/kg (free base) whereas paroxetine was injected at 20 mg/kg, both

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dissolved in 10% hydroxypropyl β-cyclodextrin/sterile saline solution (vehicle), sterile-filtered (0.2 μm syringe filter, 195−2520, Thermo-Scientific, Waltham, MA), and administered intraperitoneal (i.p.) at 10 μL/g animal weight. For chronic studies, vortioxetine was delivered in animal chow (Research Diets, New Brunswick, NJ) at 600 mg/kg chow, equating to roughly 10 mg/kg-day drug delivery of vortioxetine in vehicle chow (Purina Rodent Diet # 5001).

Animals. All procedures with mice were carried out under a protocol approved by the Vanderbilt University Institutional Animal Care and Use Committee (IACUC). Mice were housed on a 12:12 L/D cycle with food and water available ad libitum. SERT Met172 knock-in mice derive from a 129S6 transgenic line described previously.[26] Animals used in the present study had been backcrossed for >10 generations onto a C57BL/6 genetic background as described previously,[27] maintaining the ER coding haplotype that distinguishes 129 and C57 strains.[46] WT animals in the present study thus derive from SERT Met172 heterozygous breeders, subsequently bred as homozygotes in parallel with Met172 homozygotes.[27] Animals used in all experiments were initiated with male mice that were 8−12 weeks of age at initiation of the study.

Cellular and Synaptosomal 5-HT Uptake. *Cellular Uptake.* HEK293T cells were maintained in standard DMEM growth media and maintained at 37 °C and 5% CO_2. Cells were transfected with either hSERT of hSERTMet172 contained in pcDNA3 plasmid backbone at 500 ng DNA/well in TransIT (Mirus Inc., 3 uL/ug DNA) in OPTI-MEM in opaque 24-well culture plates (PerkinElmer, Inc.). 5-HT uptake (20 nM [^{3}H]5-HT (PerkinElmer, NET498001MC, Waltham, MA) assays were performed in KRH assay buffer 36−48 h following transfection, and quantified using MicroScint 20 (PerkinElmer) and scintillation detection by TopCount (PerkinElmer).

Synaptosomal Uptake. Midbrain synaptosomes were prepared by Teflon/glass (Wheaton, Inc., Millville, NJ) homogenization of freshly dissected brain tissue following rapid decapitation. Crude synaptosomes were isolated and resuspended as described previously.[26] Equal volumes of synaptosomes were incubated with 20 nM [^{3}H]5-HT (PerkinElmer) and varying concentrations of vortioxetine at 37 °C for 10 min. Uptake was terminated via vacuum filtration through GF/B filter (Whatman, Pittsburgh, PA) and washes with ice cold 1× PBS buffer. Specific uptake was defined in parallel assays uptake conducted in the presence of 1 μM paroxetine.

Ex Vivo SERT Occupancy. To evaluate differences in vortioxetine occupancy of SERT in the brains of WT and SERT Met172 in vivo, we administered the drug or vehicle (10% hydroxypropyl β-cyclodextrin in water) at 5 and 10 mg/kg i.p. mice 1 h prior to sacrifice. Following decapitation, brains were removed, frozen on dry ice and stored at −20 °C until further use. Frontal cortex was coronally sliced at 20 μm thickness approximately 0.9−0.7 mm anterior from Bregma (Paxinos and Franklin, 2004) using a cryostat (Microm, Walldorf, Germany), and mounted onto slides. Slides were stored in slide boxes with desiccator pellets at −20 °C until use. Slide boxes were allowed to defrost for 30 min at room temperature under a high flow air stream prior to opening. Slides were incubated in assay buffer optimized for SERT occupancy determination[47] (50 mM Tris HCl, 120 mM NaCl, and 5 mM KCl (pH = 7.4)) containing 1 nM [^{3}H]paroxetine for 2 h at room temp. Paroxetine was used to label SERT due to its high-affinity interactions and insensitivity to the SERT Met172 substitution.[25−27] Nonspecific binding was determined in parallel via incubation in excess (1 μM) nonradiolabeled paroxetine in assay buffer. Slides were then washed twice in room temperature assay buffer for 30 min. Slides were allowed to air-dry following washes for 30 min before transferal to a vacuum desiccator for at least 1 h. Quantitation of section bound [^{3}H]paroxetine was performed using a Beta Imager (Biospace Lab, Paris, France) after 24 h of exposure, and quantified using β-Vision software (Biospace lab). Specific binding (cpm/mm^2) was determined for each mouse brain by subtracting from total binding the average nonspecific binding, obtained from multiple, a priori defined sites that include the medial septum, lateral septum, and olfactory tubercle. Fractional occupancy in sections from vortioxetine-injected mice was determined relative to the specific binding achieved in animals given vehicle injections.

In Vivo Chronoamperometry. Vortioxetine effects on in vivo 5-HT clearance were assessed by chronoamperometry using 30 μM carbon fiber electrodes as previously described.[26] Briefly, mice were anesthetized by i.p. injection (2 mL/kg body weight) of a mixture of chloralose (35 mg/kg) and urethane (350 mg/kg). Prior to recordings, a tube was inserted into the trachea to facilitate breathing and body temperature was maintained at 36 to 37 °C by a water-circulated heating pad. The electrode−micropipette recording assembly was stereotaxically lowered into the CA3 region of the dorsal hippocampus [anteroposterior (AP), −1.94 from Bregma; mediolateral (ML), +2.0 from midline; dorsoventral (DV) −2.0 from dura]. To assess 5-HT clearance kinetics, 5-HT was pressure ejected in increasing volumes to attain signal amplitudes matching in vitro calibration standards of approximately 0.5 μM. To examine the effect of vortioxetine on 5-HT clearance, exogenous 5-HT was intrahippocampally applied by pressure-ejection before and after i.p. injection of 10 mg/kg vortioxetine, with T_{80} values, the time it takes to clear 80% of applied 5-HT, collected at 10 min intervals.

In Vivo Microdialysis. To evaluate the impact of vortioxetine on extracellular 5-HT levels, we assessed 5-HT levels via in vivo microdialysis as previously described.[26] Briefly, a guide cannula was implanted above the ventral hippocampus (stereotactic coordinates for the tip of the guide cannula were −3.18 AP, 2.8 ML, and −1 DV, relative to Bregma). At 24 h after recovery from surgery, a microdialysis probe (3 mm active site, 20 000 Da cutoff, from Synaptech, MI) was inserted into the guide cannula and perfused with aCSF (149 mM NaCl, 2.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, pH 7.2) at a flow-rate of 1 μL/min. aCSF samples were collected in 20 min intervals before and after 10 mg/kg vortioxetine (i.p.). 5-HT levels were quantified by HPLC/EC in the Vanderbilt Brain Institute Neurochemistry Core. Probe location was verified post-mortem via cresyl violet staining.

Behavioral Studies. All behavioral assays were performed in the Vanderbilt Brain Institute Murine Neurobehavioral Core Laboratory. Animals were housed within the facility for 1 wk prior to behavioral manipulations. For acute behavioral studies, drugs were prepared fresh and dissolved in 10% β-cyclodextrin/sterile saline solution and injected i.p. at 10 μL/g body weight. For chronic studies, we administered vortioxetine in specially formulated rodent chow to accomplish CNS drug levels equivalent to an ∼10 mg/kg-day i.p. dosing regimen. Drug-containing chow was provided for 28 days prior to behavioral screening.

Tail Suspension Test (TST). Mice were injected (i.p) with saline vehicle, vortioxetine (10 mg/kg), or paroxetine (20 mg/kg) 60 min before a 6 min TST.[48] Mice were then suspended by securing the tail to a vertical aluminum bar and activity was recorded by video. Immobility was defined as mice being motionless, excluding minute limb movements. Time immobile was assessed manually from video recordings by an observer blinded to genotype and drug treatment.

Forced Swim Test (FST). Mice were injected (i.p) with saline vehicle, vortioxetine, or paroxetine 60 min before a 6 min FST.[49] Mice were placed in the center of a 15 cm diameter clear plexiglass cylinder filled with tap water (25−27 °C) to a depth of approximately 15 cm and the 6 min FST was recorded by video for subsequent behavioral analysis. Immobility was defined as when mice only made movements to remain floating. Time immobile was tabulated manually by an observer blinded to genotype and drug treatment.

Novelty Induced Hypophagia (NIH). For the NIH test,[35] animals were trained daily in 30 min sessions for 3 days to consume a palatable substance (Vanilla Ensure) in their home cage under nonaversive, low red light conditions (∼50 lm). On the first day of testing, mice were moved to a novel cage with no bedding and aversive, high intensity white light illumination (∼1200 lm), where the latency to first consume Vanilla Ensure was measured. On the following day, latency values of Vanilla Ensure were assessed in the home cage under low light conditions. Animals were singly housed during training and testing phases.

Hippocampal Neurogenesis. The generation and survival of subgranular dentate gyrus stem cells was assessed as previously described.[27,34] Mice were given normal or vortioxetine-containing

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chow for 28 days and newly born hippocampal stem cells were detected by immunohistochemistry. Newly proliferating S-phase stem cells were pulse labeled in vivo using 5-bromo-2′-deoxyuridine (BrdU; 150 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO) following or prior to chronic administration of vortioxetine to measure hippocampal stem cell proliferation or survival, respectively.

Proliferation. Following chronic administration of antidepressants and behavioral screening in the NIH test, mice were administered BrdU as noted above. At 24 h following the injection of BrdU, mice were anesthetized via injection of 100 mg/kg i.p. pentobarbital and transcardially perfused with ice-cold PBS, followed by ice-cold 4% paraformaldehyde. Brains were sectioned (40 μm) using a freezing stage sliding microtome (Leica, SM2000R, Buffalo Grove, IL). Every sixth section of the hippocampus (plates 41−61 (Paxinos and Franklin, 2004)) was immunostained for BrdU incorporation (mouse anti-BrdU; 1:1000; BD#347580; BD Biosciences; Franklin Lakes, NJ) and detected following secondary antibody incubation (biotinylated donkey anti-mouse; 1:500; PA1-28627; ThermoFisher), ABC amplification (VectaStain; Vector Laboratories; Burlingame, CA), and diaminobenzidine (DAB) detection. Brightfield stitched images were captured (Zeiss Axio Imager.M2) and stored for analysis. BrdU+ cells in the subgranular zone (SGZ) of the hippocampus were counted using the ITCN (Image-based Tool for Counting Nuclei) plugin for ImageJ[50] by an observer blinded to genotype and drug treatment. Total counts were extrapolated to whole hippocampus, accounting for initial sampling limits.

Survival. To assess stem cell survival, we injected a separate cohort with BrdU (150 mg/kg i.p.) 24 h prior to administration of antidepressants, as described above. After 4 weeks of antidepressant administration, mice were sacrificed and brains were collected and sectioned, and BrdU+ cells were detected and quantitated as described above.

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A.G.N., R.D.B., and C.S. designed the study. A.G.N., L.D.S., N.L.B., and A.L.P. performed experiments and analyzed data. All authors participated in the writing of the paper.

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Notes

C.S. and A.L.P. were employees of Lundbeck, USA during the execution of the study.

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