

The SSRI Citalopram Affects Fetal Thalamic Axon Responsiveness to Netrin-1 *In vitro* Independently of SERT Antagonism

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Serotonin (5-hydroxytryptamine, 5-HT) signaling is thought to modulate nervous system development. Genetic and pharmacological studies support the idea that altered 5-HT signaling during development can have enduring consequences on brain function and behavior. Recently, we discovered that 5-HT can modulate thalamic axon guidance *in vitro* and *in vivo*. Embryonic thalamic axons transiently express the 5-HT transporter (SERT; *Slc6a4*) and accumulate 5-HT, suggesting that the SERT activity of these axons may regulate 5-HT-modulated guidance cues. We tested whether pharmacologically blocking SERT using selective 5-HT reuptake inhibitors (SSRIs) would impact the action of 5-HT on thalamic axon responses to netrin-1 *in vitro*. Surprisingly, we observed that two high-affinity SSRIs, racemic citalopram ((*RS*)-CIT) and paroxetine, affect the outgrowth of embryonic thalamic axons, but differ with respect to their dependence on SERT blockade. Using a recently developed 'citalopram insensitive' transgenic mouse line and *in vitro* pharmacology, we show that the effect of (*RS*)-CIT effect is SERT independent, but rather arises from *R*-CIT activation of the orphan sigma-1 receptor ($\sigma 1$, *Opr1*). Our results reveal a novel $\sigma 1$ activity in modulating axon guidance and a 5-HT independent action of a widely prescribed SSRI. By extension, (*RS*)-CIT and possibly other structurally similar SSRIs may have other off-target actions that can impact neural development and contribute to therapeutic efficacy or side effects.

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INTRODUCTION

Genetic studies in mice show that disruption of 5-hydroxytryptamine (5-HT) signaling during a restricted period of pre- and postnatal development results in long-term behavioral abnormalities, such as increased anxiety in adulthood (Gaspar *et al*, 2003; Oberlander *et al*, 2009). Interestingly, the fetal programming of adult anxiety can be triggered either by a transient knockdown of a single 5-HT receptor (5-HT_{1A}, (Gross *et al*, 2002)) during the pre- and early postnatal periods or by a transient developmental exposure to SSRIs (Ansorge *et al*, 2004; Ansorge *et al*, 2008). Moreover, the forebrain acquires placenta-derived 5-HT during a period of substantial axon outgrowth (Bonnin *et al*, 2011), for example of thalamocortical axons

(Lopez-Bendito and Molnar, 2003). This suggests that the control of 5-HT signaling, either through the expression and activity of 5-HT receptors or through extracellular 5-HT availability, is critical for normal brain development. *In vitro*, we demonstrated that 5-HT signaling through 5-HT_{1B/1D} receptors switches the response of thalamic axons to netrin-1 from attraction to repulsion, mediated by a cAMP-dependent pathway (Bonnin *et al*, 2007). Furthermore, disruption of 5-HT_{1B/1D} receptor expression in the dorsal thalamus by *in utero* electroporation at embryonic (E) 12.5 leads to abnormal navigation of thalamocortical axons through the internal capsule and cortex (Bonnin *et al*, 2007).

Interestingly during embryonic and early postnatal development, thalamocortical axons transiently express SERT (Lebrand *et al*, 1996; Bruning and Liangos, 1997; Bruning *et al*, 1997; Lebrand *et al*, 1998; Narboux-Neme *et al*, 2008). The SERT-mediated uptake of 5-HT in thalamic axons has been shown to influence the precision of cortical barrel map formation (Lebrand *et al*, 1996; Persico *et al*, 2001). However, during the early phase of fetal thalamocortical axon growth, the role of SERT is not known. Based on 5-HT signaling effects on thalamic axons guidance, we hypothesized

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that SERT-mediated uptake could restrict availability of extracellular 5-HT levels in the vicinity of growing axons and therefore affect the amplitude of 5-HT modulation netrin-1 signaling. A prediction of this hypothesis is that blockade of SERT in growing thalamic axons *in vitro* should decrease the minimal concentration of extracellular 5-HT needed to switch axonal responses to netrin-1 from attraction to repulsion.

Therefore, we sought to compare the effects of increasing concentrations of 5-HT on the response of thalamic axons to netrin-1 in the absence and presence of a potent SSRI, (RS)-CIT. Surprisingly, (RS)-CIT, but not the SSRI paroxetine, switched thalamic axons response to netrin-1 in the absence of extracellular 5-HT. Moreover, thalamocortical axons generated from transgenic mice that lack high-affinity (RS)-CIT recognition by SERT remained sensitive to the SSRI. We further show that (RS)-CIT effects arise via R-CIT activation of the high-affinity σ 1 receptor (Su, 1982; Narita *et al*, 1996).

MATERIALS AND METHODS

Animals and Reagents

Timed-pregnant C57BL/6J and CD-1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Plug date was considered E0.5 and the age of individual embryos confirmed by measuring the crown-rump length and checking for developmental landmarks such as digits and eye formation. The production and characterization of SERT M172 transgenic mice were described earlier (Thompson *et al*, 2011). SERT M172 homozygous embryos were obtained by crossing homozygous males and females. This line has been backcrossed on the C57BL/6J background for more than 10 generations. All research procedures using mice were approved by the Institutional Animal Care and Use Committee at University of Southern California and conformed to NIH guidelines. Unless otherwise noted, all reagents were purchased from Sigma (St Louis, MO, USA).

Explant Assays

We used a coculture assay to monitor axonal growth from embryonic thalamic explants toward or away from a source of soluble guidance cues (HEK-293 cells stably expressing netrin-1 or slit-2—gift from J Wu (Northwestern University)). The procedure, quantification methods, and statistical analysis were previously described in detail in Bonnin (2010) and Bonnin *et al*, (2007). The explants were coded so that the investigators performing the quantitative analyses of axon growth were blinded to the specific treatments.

Immunohistochemistry

Brains ($n=3$) were harvested from E16.5 embryos and immersion-fixed overnight at 4°C in phosphate-buffered 4% paraformaldehyde (PFA; pH 7.2). Following cryoprotection in sucrose-phosphate buffer, sagittal cryostat sections (20 μ m) were collected for staining. PFA-fixed explants (15 min) were incubated overnight in primary antibody (2% BSA, 0.2% Tween-20 in PBS) using the following dilutions: anti- σ 1, 1:500 (kind gift from Dr Su (NIH/DHHS)); Tuj1, 1:500 (Covance). For sections, the primary antibodies used were: rabbit anti-SERT (Sigma; 1:200) and goat anti-Netrin

G1a (NetG1a, RnD; 1:250). NetG1a is a marker of fetal thalamocortical axons (Nakashiba *et al*, 2000; Bonnin *et al*, 2011). Sections and explants were washed extensively, incubated overnight with cy²³-conjugated secondary antibodies (Jackson Immunoresearch, 1:1000), washed, and for cryostat sections, embedded in Prolong Gold with DAPI (Invitrogen) and imaged using an Axiocam CCD camera coupled to a Leica MZFLIII stereoscope and an Olympus confocal microscope.

RT-PCR

Primers used for σ 1 receptor cDNA detection, using 30 PCR cycle amplification, were as follows: reverse: 5'-ACGGAA TAACACCCCGGCCGT-3'; forward: 5'-TTCTGCACGCCTC GCTGTCTG-3'. Primers span an 1102-bp intron of the σ 1 receptor gene (Mus musculus sigma non-opioid intracellular receptor 1; Accession #: NM_011014) and therefore the 255-bp amplicon can only result from PCR amplification of the σ 1 cDNA.

RESULTS

In order to explore the actions of 5-HT and SERT on axon outgrowth *in vitro*, we used a previously described E14 thalamic explant assay (Braisted *et al*, 2000; Bonnin *et al*, 2007; Bonnin, 2010). This method allows the monitoring of axon responses to the soluble guidance netrin-1 in a three-dimensional matrix. We first established a dose response of 5-HT effect on thalamic axons response to netrin-1, using concentrations ranging from 3 nM to 30 μ M, the latter being a concentration that we showed can switch netrin-1 attraction to repulsion (Bonnin *et al*, 2007). Replicating our previous study (Bonnin *et al*, 2007), 5-HT significantly affected thalamic axon response to netrin-1 at concentrations equal to or greater than 30 nM in the culture medium (Figure 1a). The lowest concentration of 5-HT tested (3 nM) had no significant effect on directional growth. We then tested whether a high-affinity blocker of SERT-mediated 5-HT uptake could decrease the minimal concentration of extracellular 5-HT capable of switching their response to netrin-1. RT-PCR and immunostaining demonstrated that SERT is expressed by thalamic axons at E14 and E16 (Figures 1c–f; Bruning and Liangos, 1997; Bruning *et al*, 1997; Lebrand *et al*, 1998). To block thalamocortical axon 5-HT uptake *in vitro*, we performed a 5-HT dose-response assay in the presence of the potent SERT antagonist (RS)-CIT (10 μ M). As expected, in the presence of (RS)-CIT the lowest concentration of 5-HT (3 nM) was able to affect thalamic axons responses to netrin-1 (Figure 1b). Unexpectedly, however, (RS)-CIT alone was equally capable of switching the response of thalamic axons to netrin-1 from attraction to repulsion (Figure 1b), even in the absence of extracellular 5-HT. In separate (RS)-CIT dose-response experiments, we observed that citalopram concentrations as low as 30 nM were sufficient to significantly affect thalamic axons response in the absence of extracellular 5-HT (not shown).

These data suggest that blocking SERT in thalamic axons triggers a change in responsiveness to netrin-1, even in the absence of extracellular 5-HT-mediated signaling. Alternatively, (RS)-CIT could directly affect axons behavior independently of its binding to SERT. In order to test this

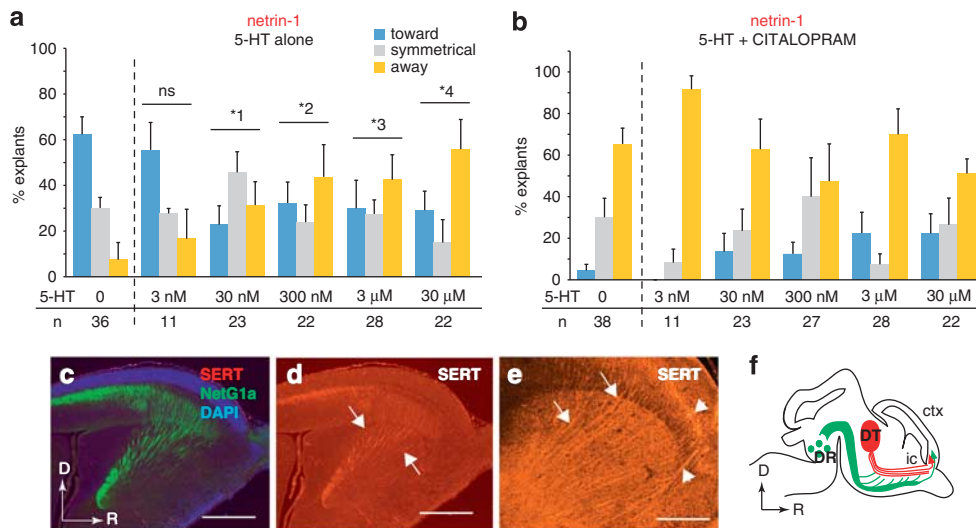


Figure 1 Dose-response analyses of 5-hydroxytryptamine (5-HT) and 5-HT+(*RS*)-CIT on the response of thalamic axons to netrin-1 *in vitro*. (a) Quantification over four independent experiments shows that the minimal concentration of 5-HT capable of switching the response of posterior thalamic axons to netrin-1 from attraction to repulsion is 30 nM (*1, $\chi^2 = 34.83$, *2, $\chi^2 = 35.16$, *3, $\chi^2 = 35.31$, *4, $\chi^2 = 52.97$, $P < 0.0001$, $df = 2$; ns, not significant, $\chi^2 = 3.61$, $P = 0.1645$, $df = 2$; χ^2). (b) In the presence of (*RS*)-CIT (10 μ M), all concentrations of 5-HT switched the response of netrin-1 exposed thalamic axons to repulsion, similar to the effect of (*RS*)-CIT alone. *n*, number of explants tested in each condition. (c, d) Immunostaining on sagittal sections showing that E16.5 thalamocortical axons (NetG1a+) express SERT (white arrows); D = dorsal, R = rostral. Scale bars = 1 mm. (e) Higher magnification of the striatum and internal capsule region showing SERT+ thalamic axons (white arrows) and SERT+ serotonergic axons arising from the dorsal raphe (white arrowheads). Scale bar = 0.5 mm. (f) Schematics showing the pathways of both populations of SERT+ axons (thalamic, red and serotonergic, green) growing through the forebrain at E16.5.

possibility, we took advantage of a recently developed transgenic mouse line, mSERT M172; these mice possess a modified copy of SERT with a single amino-acid substitution, I172M, proximal to the 5-HT-binding site (Henry *et al*, 2006; Thompson *et al*, 2011). The M172 substitution does not impact the recognition or transport of 5-HT, but disrupts high-affinity binding of many SSRIs. Importantly, the M172 substitution confers an ~ 1000 -fold reduction in potency for (*RS*)-CIT but not paroxetine, another SSRI at mSERT (Henry *et al*, 2006), and mice bearing the substitution display insensitivity to (*RS*)-CIT *in vivo*. Therefore, we compared the effect of 5-HT (30 μ M), (*RS*)-CIT (10 μ M), and paroxetine (10 μ M) on the response of thalamic axons to netrin-1 using explants generated from homozygous mSERT M172 mice or wild-type embryos. As previously observed (Figure 1a), 5-HT and (*RS*)-CIT switched the attractive effect of netrin-1 on thalamic axons from wild-type embryos, but paroxetine did not (Figure 2a). Furthermore, in cultures generated from SERT M172 embryonic tissue, 5-HT and (*RS*)-CIT still switched the attractive effect of netrin-1 on thalamic axons, and paroxetine again had no effect (Figure 2b). These data strongly argue that (*RS*)-CIT effects are not mediated by SERT antagonism.

An alternative target of (*RS*)-CIT is the $\sigma 1$ receptor (Su, 1982; Narita *et al*, 1996; Sanchez and Meier, 1997). Like several other SSRIs, (*RS*)-CIT can act as an agonist of $\sigma 1$ receptors, displaying an affinity of ~ 2 –300 nM for this binding site; in contrast, paroxetine shows a much lower affinity (~ 1900 nM) (Narita *et al*, 1996; Sanchez and Meier, 1997). $\sigma 1$ receptor transcripts are expressed in the dorsal thalamus at E14 and E16 (Figure 3d), and the presence of receptor proteins was detected along thalamic axons growing *in vitro* (Figure 3e). In order to test whether $\sigma 1$ mediates the (*RS*)-CIT effect on the response of thalamic axons to

netrin-1, we co-incubated the cultures with (*RS*)-CIT (10 μ M) and BD-1047 (1 μ M), a potent $\sigma 1$ receptor antagonist (Maurice and Su, 2009). Results showed that BD-1047 blocked the effect of (*RS*)-CIT (Figure 3a). The antagonistic effect of BD-1047 on (*RS*)-CIT action could be observed with concentrations as low as 10 nM (not shown). BD-1047 by itself had no effect (Figure 3b). Interestingly, the potent $\sigma 1$ receptor agonist PPBP (1 μ M) switched thalamic axons response to netrin-1 from attraction to repulsion, similar to the effect of (*RS*)-CIT (Figure 3b). Given that (*RS*)-CIT is acting in a SERT-independent manner on axon guidance and that the enantiomer *R*-CIT displays a 100-fold shift in potency at SERT 172M (Henry *et al*, 2006), the *R*-isomer could be preferentially acting at the $\sigma 1$ receptor. We therefore tested the effects of *R*- and *S*-CIT on axon guidance independently. Results showed that *R*-CIT, but not *S*-CIT, switched thalamic axons response to netrin-1 (Figure 3c); furthermore, BD-1047 blocked the effect of *R*-CIT (Figure 3c).

DISCUSSION

The data presented here reveal an unexpected, direct effect of the SSRI (*RS*)-CIT on embryonic thalamic axons response to the guidance cue netrin-1 *in vitro*. Our initial hypothesis, based on 5-HT signaling effects on thalamic axons guidance (Bonnin *et al*, 2007), was that SERT-mediated uptake could control extracellular 5-HT levels in the vicinity of growing axons and therefore affect the amplitude of 5-HT modulation of their response to netrin-1. Therefore, we tested if blocking 5-HT uptake with SSRIs in growing thalamic axons decreases the minimal concentration of extracellular 5-HT capable of switching their response to netrin-1 from attraction to repulsion *in vitro*. We observed surprising

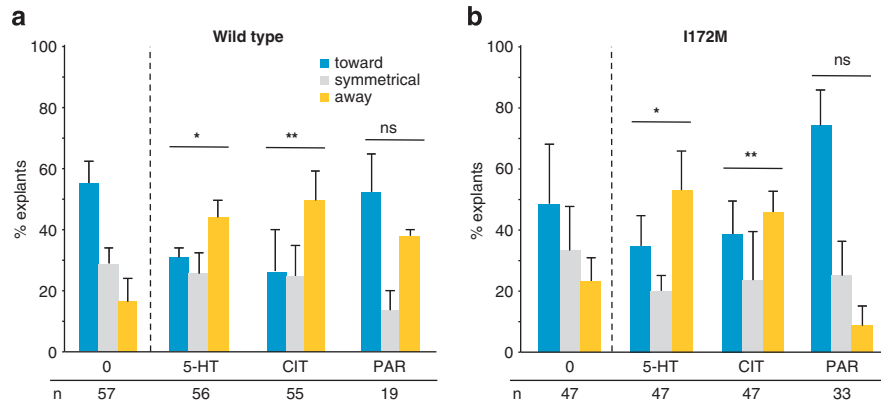


Figure 2 Effect of (*RS*)-CIT in thalamic explants from wild-type and SERT M172 mice. (a) 5-hydroxytryptamine (5-HT) ($30 \mu\text{M}$) or (*RS*)-CIT (CIT, $10 \mu\text{M}$) significantly affect the response of wild-type thalamic axons to netrin-1 (*, $\chi^2 = 20.06$; **, $\chi^2 = 27.43$, $P < 0.0001$, $df = 2$; χ^2). The effect remains in cultures from SERT M172 (b) embryos, where (*RS*)-CIT affinity for SERT is decreased (~ 1000 fold) compared with wild-type (*, $\chi^2 = 21.86$; **, $\chi^2 = 18.90$, $P < 0.0001$, $df = 2$; χ^2 ; ns, not significant, $\chi^2 = 5.2$, $P = 0.0743$, $df = 2$; χ^2). In both cultures, the SSRI paroxetine (PAR, $10 \mu\text{M}$) has no significant effect. n, number of explants tested in each condition.

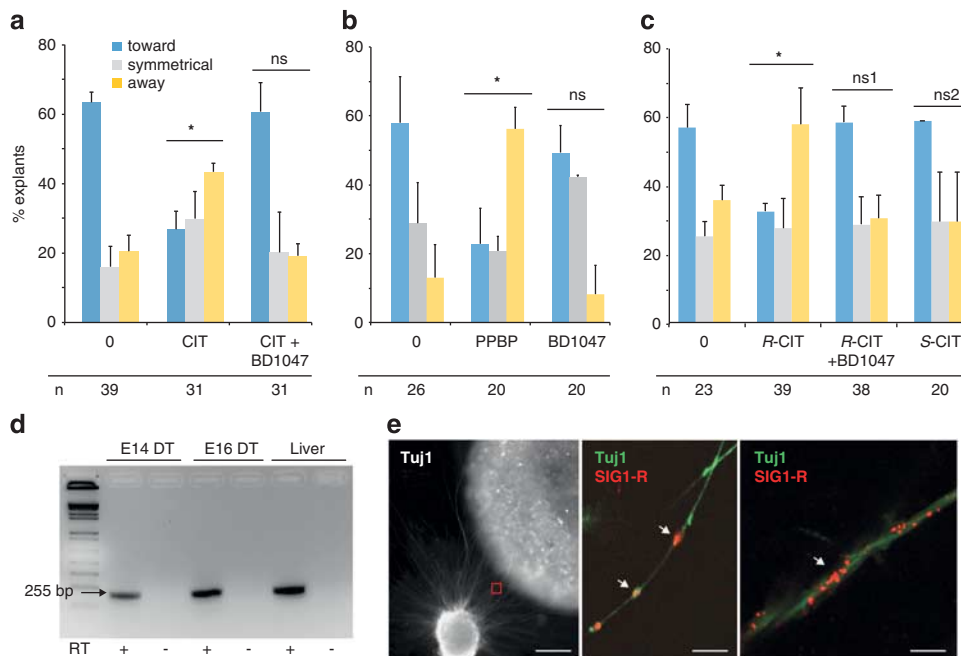


Figure 3 The effect of (*RS*)-CIT is mediated by the $\sigma 1$ receptor. (a) (*RS*)-CIT (CIT, $10 \mu\text{M}$) significantly alters the response of wild-type thalamic axons to netrin-1. The effect is blocked by co-incubation with the $\sigma 1$ antagonist BD-1047 (CIT + BD-1047; CIT, $10 \mu\text{M}$; BD-1047, $1 \mu\text{M}$; *, $\chi^2 = 26.22$, $P < 0.0001$, $df = 2$; χ^2 ; ns, not significant, $\chi^2 = 0.57$, $P = 0.7495$, $df = 2$; χ^2). (b) BD-1047 alone has no effect, but the $\sigma 1$ agonist PPBP ($1 \mu\text{M}$) switches the response of thalamic axons to netrin-1, similar to the effect of citalopram (*, $\chi^2 = 43.20$, $P < 0.0001$, $df = 2$; χ^2 ; ns, not significant, $\chi^2 = 4.16$, $P = 0.1247$, $df = 2$; χ^2). (c) (*R*)-CIT (R-CIT, $10 \mu\text{M}$), but not (*S*)-CIT ($10 \mu\text{M}$), significantly alters the response of thalamic axons to netrin-1. The effect is blocked by co-incubation with the $\sigma 1$ antagonist BD-1047 (R-CIT + BD-1047; R-CIT, $10 \mu\text{M}$; BD-1047, $1 \mu\text{M}$; *, $\chi^2 = 9.85$, $P = 0.0072$, $df = 2$; χ^2 ; ns1, not significant, $\chi^2 = 0.41$, $P = 0.8132$, $df = 2$; χ^2 ; ns2, not significant, $\chi^2 = 0.68$, $P = 0.7093$, $df = 2$; χ^2). n indicates the number of explants tested in each condition. (d) PCR amplification of dorsal thalamus cDNA shows that $\sigma 1$ is expressed in the structure at E14 (E14 DT) and E16 (E16 DT); cDNA from the adult liver was used as a positive control for the reaction (liver). RT +/- indicates the presence (+) or absence (-) of reverse transcriptase during the cDNA synthesis reaction. (e) The $\sigma 1$ receptor protein is expressed along thalamic axons growing *in vitro*. Immunostaining was performed on thalamic explants (E14) grown *in vitro* for 3 days in apposition to HEK cells expressing netrin-1 (left panel). Right panel is a high magnification of the boxed area on the left panel. White arrows point to varicose-like structures showing intense $\sigma 1$ labeling. Scale bars = $400 \mu\text{m}$ (left panel), 10 and $5 \mu\text{m}$ (right panels, respectively).

activity of (*RS*)-CIT alone, in the absence of extracellular 5-HT in the culture medium, affecting the response of thalamic axons to netrin-1; this suggested that SERT antagonism might be responsible for the effect, independent of 5-HT transport. Previous studies showed that SERT supports substrate-independent transient conductance in developing

thalamocortical axons *in vitro*, which could affect cellular activity and can be blocked by antagonists (Quick, 2002, 2003). In order to test whether the (*RS*)-CIT effect was mediated by blocking substrate-independent SERT activity, we measured the influence of the drug on the response of axons to netrin-1 using thalamic explants derived from

mSERT M172 mice. Although (RS)-CIT shows ~1000-fold reduction in affinity at the SERT-binding site in the transgenic mice (Henry *et al*, 2006; Thompson *et al*, 2011), the drug still induced a significant change in thalamic axon responsiveness to netrin-1, supporting the idea that (RS)-CIT effects are likely mediated independently of SERT. Interestingly, studies have shown that several SSRIs, including fluvoxamine and citalopram, can act as agonists of σ 1 receptors (Narita *et al*, 1996; Sanchez and Meier, 1997; Maurice and Su, 2009). Consistent with this possibility, the effect of (RS)-CIT was blocked by the σ 1 receptor antagonist BD-1047. Additionally, (RS)-CIT effects were mimicked by the σ 1 receptor agonist PPBP. Interestingly, it was shown that the enantiomer R-CIT displays a 100-fold shift in potency at SERT 172M (Henry *et al*, 2006), suggesting that neither R- or S-CIT would be working through SERT to modulate axon guidance, as indicated by the use of racemic citalopram. However, given that (RS)-CIT is acting in a SERT-independent manner on axon guidance, this raises the possibility that the R-isomer could be preferentially acting at the σ 1 receptor. Consistent with this possibility, R-CIT alone was capable of switching thalamic axons response to netrin-1, and the σ 1 receptor antagonist BD-1047 blocked this effect. Furthermore, we tested the actions of the SSRI paroxetine, which has higher affinity for SERT than citalopram (Henry *et al*, 2006) but is not an agonist of σ 1 receptors (Nishimura *et al*, 2008; Hashimoto, 2010). Importantly, paroxetine retains full potency at mSERT M172 (Henry *et al*, 2006). In keeping with the SERT- and 5-HT independence of SSRI action in our assays, paroxetine had no effect on thalamic axon responses, either in the wild-type or mSERT I172M explants. Using RT-PCR, we confirmed that σ 1 receptor is expressed in the developing thalamus at ages used to generate explant cultures (E14 to E16), and interestingly, the protein appeared localized in discrete, varicose-like, regions along thalamic axons growing *in vitro*.

Although the mechanism by which σ 1 receptor activation affects axons response to netrin-1 remains to be investigated, previous studies suggest several potential pathways; for instance, stimulation of σ 1 receptors with fluvoxamine, which potentiates nerve-growth factor-induced neurite outgrowth in PC 12 cells, is mediated by σ 1 receptor interaction with IP(3) receptors, PLC-gamma, PI3K, p38MAPK, JNK, and the Ras/Raf/MAPK signaling pathways (Takebayashi *et al*, 2002; Su *et al*, 2010). Each of these pathways contributes to axon guidance mechanisms (Bashaw and Klein, 2010). Interestingly, σ 1 receptor has been localized to the endoplasmic reticulum (ER) membrane (Mavlyutov *et al*, 2010; Su *et al*, 2010) and can modulate cell membrane excitability by regulating the activity of several ion channels, including intracellular Ca^{2+} channels (Hayashi and Su, 2007); changes in intracellular Ca^{2+} concentration is a well-known modulator of axonal responses to guidance cues (Hong *et al*, 2000; Xiang *et al*, 2002; Nishiyama *et al*, 2003; Wang and Poo, 2005). Another intriguing possibility, related to σ 1 receptor presence on ER membranes, is a direct effect on guidance cue receptors localization at the plasma membrane. Studies have shown that axonal ER entry sites (ERES) may be used to facilitate axon guidance by regulating the delivery of proteins such as the EphA2 receptor to the plasma membrane (Martin, 2004; Aridor and Fish, 2009). Interestingly, the expression pattern of ERES

protein Sar1 along growing axons *in vitro* (Aridor and Fish, 2009) shows striking similarities with that of σ 1 receptor described here. Thus, a testable hypothesis is that citalopram and other σ 1 receptor agonists could affect netrin-1 receptors (eg, DCC and Unc5c) delivery to the plasma membrane along thalamic axons and in growth cones. Similar to previously described regulation of DCC translocation to the cell surface by changes in intracellular cAMP, such ER/ σ 1-mediated receptor delivery modulation could affect axons response to netrin-1 (Bouchard *et al*, 2004; Moore *et al*, 2008). *In vivo*, citalopram effect on SERT would concurrently raise extracellular 5-HT concentration potentially leading to convergence of increased signaling through 5-HT_{1B/1D} receptors (Bonnin *et al*, 2007) and σ 1 receptors, which both induce switching of thalamic axons response to netrin-1 (Bonnin *et al*, (2007) and present results).

Although an effect of citalopram on axon guidance *in vivo* through σ 1 receptors must now be demonstrated, our results suggest that *in utero* exposure of the fetal forebrain to this SSRI could affect neural development, independent of the effects of manipulating 5-HT signaling *in vivo* (Bonnin *et al*, 2007). Citalopram crosses the placental barrier (Hendrick *et al*, 2003) in humans, raising the possibility that this agent, in particular the R-isomer, and its congeners may have unintended consequences on fetal brain development. Mood disorders themselves place the mother and fetus at risk (Casper *et al*, 2003; Yonkers *et al*, 2009), and our studies cannot serve to predict the risk/benefit aspects of SSRI treatments during pregnancy. Further studies are needed to determine whether σ 1 receptor-mediated actions participate in the therapeutic or side effects of antidepressant treatment.

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DISCLOSURE

RDB is on the Scientific Advisory Board of Lundbeck, makers of citalopram and escitalopram, but the work presented here was not conceived or supported by Lundbeck. The other authors declare no conflict of interest.

Author Contributions

AB designed and performed the research, analyzed the data, and wrote the paper. LZ performed the research. RDB wrote the paper. PL designed the research, analyzed the data, and wrote the paper.

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