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Novel Role of CXCR2 in Regulation of γ-Secretase Activity

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ABSTRACT Alzheimer's disease (AD) is a progressive chronic disorder that leads to cognitive decline. Several studies have associated up-regulation of some of the chemokines and/or their receptors with altered APP processing leading to increased production of β-amyloid protein (Aβ) and AD pathological changes. However, there is no direct evidence to date to determine whether the altered processing of APP results in up-regulation of these receptors or whether the up-regulation of the chemokine receptors causes modulated processing of APP. In the current study, we demonstrate that treatment of the chemokine receptor CXCR2 with agonists leads to enhancement of Aβ production and treatment with antagonists or immunodepletion of CXCR2's endogenous agonists leads to Aβ inhibition. Further, we found that the inhibitory effect of the antagonist of CXCR2 on Aβ40 and Aβ42 is mediated *via* γ-secretase, specifically through reduction in expression of presenilin (PS), one of the γ-secretase components. Also, *in vivo* chronic treatment with a CXCR2 antagonist blocked Aβ40 and Aβ42 production. Using small interfering RNAs for CXCR2, we further showed that knockdown of CXCR2 *in vitro* accumulates γ-secretase substrates C99 and C83 with reduced production of both Aβ40 and Aβ42. Taken together, these findings strongly suggest for the first time that up-regulation of the CXCR2 receptor can be the driving force in increased production of Aβ. Our findings unravel new mechanisms involving the CXCR2 receptor in the pathogenesis of AD and pose it as a potential target for developing novel therapeutics for intervention in this disease. Also, we propose here a new chemical series of interest that can serve as a prototype for drug development.

Alzheimer's disease (AD) is the most common neurodegenerative disorder, characterized by memory impairments and progressive dementia. The key pathological features associated with AD include extracellular accumulation of Aβ (β-amyloid) in plaques, intracellular accumulation of the microtubule-associated protein τ in neurofibrillary tangles, inflammation, neuron loss and synapse loss (*1*–*3*). The dominantly inherited mutations in the amyloid precursor protein (APP) gene and in the presenilin 1 and 2 (PS1 and PS2) genes precipitate early onset disease (*1*). Presenilins (PS), with three other essential components (anterior pharynx defective 1 (Aph-1), presenilin enhancer 2 (Pen-2), and nicastrin (NCT)), form the multiprotein protease complex γ-secretase (*4, 5*), which catalyzes the final cleavage of APP to Aβ. The etiology of AD is not clearly defined, but the leading hypothesis is that Aβ accumulation triggers neurotoxic and neuroinflammatory events leading to abnormal τ phosphorylation and neuronal death (*6*). Although AD has not been traditionally regarded as an inflammatory process, there is an innate inflammatory reaction in the affected tissues (*7*). The most important elements of this reaction are the activation of microglia with the production of cytokines and the activation of complementary intracellular signaling pathways (*7*). A number of chemokines and their cognate receptors are expressed or up-regulated in the AD brain (*8, 9*). Immunohistochemical analysis has demonstrated significant differences in the expression of CXCR2, CXCR3, CXCR4, CCR3, and CCR5 in normal and AD brains (*8*). In parallel, a number of chemokines such as CCL2, CCL3, CCL4, CCL5, CCL8, CXCL8 (IL8), and CXCL10 have also been reported to be up-regulated in AD brains (*8*). Although chemokines and their receptors have been reported to be expressed or up-regulated in the AD brain, less is known about their role in the pathogenesis of AD, although their chronic presence is generally

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assumed to be detrimental. To explore the link between chemokines and/or their receptors in processing of APP that could have important implications for the development of new therapeutics, we judiciously designed and screened a focused library of chemokine receptor ligands. Through this screening, we identified a small molecule, *N*-(2-hydroxy-4-nitrophenyl)-*N*′-(2-bromophenyl)urea (SB225002), known to selectively antagonize CXCR2 (the chemokine receptor or IL8R) (*10*), which inhibited both Aβ40 and Aβ42 production in a cell-based assay with half-maximal inhibitory concentrations (IC_{50}'s) of ~500 and ~800 nM, respectively, without affecting α- and β-secretases. Interestingly, we found that SB225002 alters APP processing not through direct inhibition of γ-secretase but *via* modulation of PS expression. Further, we used SB225002 and other known ligands (agonists and antagonists) of CXCR2 as a biological tool to decipher the role of CXCR2 in the processing of APP. In this report we describe our findings of a prototypic new chemical series of interest and suggest CXCR2 as an alternate therapeutic target for AD.

RESULTS AND DISCUSSION

CXCR2 Antagonist SB225002 Alters APP Processing: Reduction of Aβ40 and Aβ42.

Initially, we screened a focused library of ligands of heptahelical chemokine receptors for their inhibitory effect on the production of Aβ40. The compounds were evaluated in a cell-based assay at two different concentrations, 10 and 30 μM, in 7w cells (*11*) (Chinese hamster ovary cell line stably expressing wtAPP751), which led to the identification of a hit, a CXCR2 receptor antagonist, *N*-(2-hydroxy-4-nitrophenyl)-*N*′-(2-bromophenyl)urea (*10*) (SB225002). SB225002 (*10*) is a known potent and selective nonpeptide antagonist of CXCR2, (reported to exhibit >150-fold selectivity over CXCR1) (*10*). SB225002 (*10*) showed significant inhibition of ~85% for Aβ40 levels at 10 μM in this initial screen. The hit was further confirmed from a fresh batch and evaluated for its dose-response effect on both Aβ40 and Aβ42 production. Different concentrations (30, 100, and 300 nM and 1, 3, 10, and 30 μM) of SB225002 and *N*-[*N*-(3,5-difluorophenacetyl-L-alanyl)]-*S*-phenylglycine *t*-butyl ester (DAPT) (*12*) (a known inhibitor of γ-secretase used as a standard to account for variations among experiments and sensitivity of ELISA kits used) were tested in 7w cells (*11, 13, 14*). During 18 h of treatment, SB225002 inhibited the production of both Aβ40 (Figure 1, panel a) and Aβ42 (Figure 1, panel b) in a dose-dependent manner, with an IC_{50} of ~500 and ~800 nM, respectively. DAPT decreased Aβ40 and Aβ42 levels with IC_{50}'s of ~60 and ~80 nM, respectively, lower than IC_{50}'s reported in HEKsw cells (human embryonic kidney cell line stably expressing APP with Swedish mutation) (*12*). Interestingly, no significant change in Aβ40 and Aβ42 production (data not shown) was found with SB225002 treatment at shorter time periods of 4 and 6 h.

To rule out the possibility that the observed reduction in Aβ was cell-line-specific, we treated different doses of SB225002 as well as DAPT in the HEKsw cell line. As shown in Figure 1, panels c and d, SB225002 inhibited Aβ40 and Aβ42 in a dose-dependent manner with IC_{50}'s of 2.5 and 5−7 μM, respectively, ~5 times higher than IC_{50} in 7w cells. DAPT also showed an increase of ~4.8-fold in IC_{50} for Aβ40 (~280 nM as compared to 60 nM) and Aβ42 (~0.3−0.5 μM). These differences might be due to intrinsic cell line differences in metabolism of APP or expression levels of exogenous APP.

Further, we asked if the observed change in Aβ40 was due to reduction in its production and/or secretion. We quantified the intracellular pool of Aβ40 in both 7w cells and HEKsw cells. Both cell lines were incubated with different concentrations of SB225002 and DAPT. Intracellular Aβ40 produced by 7w cells was found to be below the detection limit of ELISA. By contrast it was detectable in HEKsw cells, and both SB225002 and DAPT (control) reduced intracellular Aβ40 in these cells in a dose-dependent manner with IC_{50}'s of ~2.5 μM and ~280 nM, respectively (Figure 1, panel e). The inhibition of both extracellular and intracellular Aβ40 with the same IC_{50} suggests that SB225002 interferes with the production of Aβ and not its secretion.

SB225002 Does Not Affect Aβ Degradation. Degradation of Aβ, if any, by SB225002 could also lead to the observed reduction in Aβ. To this end, we made different concentrations of SB225002 in conditioned media from 7w cells (to provide an exogenous source of Aβ) and incubated with Chinese hamster ovary (CHO) wt cells. No change in the amount of Aβ40 was found in SB225002-treated versus control cells (data not shown), thus ruling out any effect of SB225002 on Aβ-degrading enzymes.

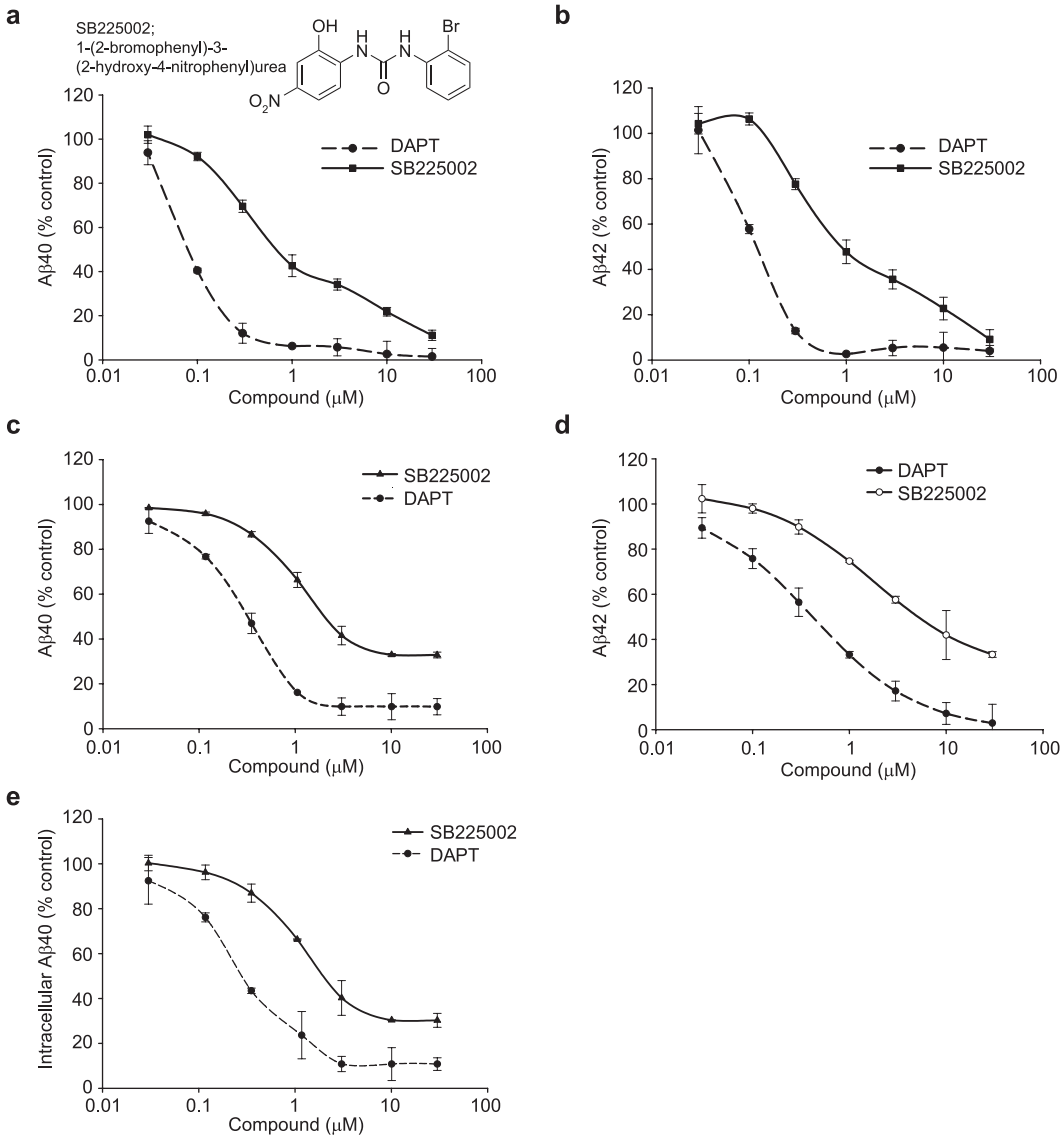

Figure 1. Effect of SB225002 on Aβ40 and Aβ42 production. a,b) IC_{50} determination of SB225002 for secreted (panel a) Aβ40 and (panel b) Aβ42 from 7w cells. The results were plotted for concentrations of SB225002 or control DAPT (a known inhibitor of γ-secretase) versus secreted Aβ40 or Aβ42 levels relative to percent control (1% DMSO). c) Aβ40 and d) Aβ42 secreted from HEKsw cells after treatment with different doses of SB225002 or DAPT was calculated relative to percent control (1% DMSO). e) Protein-normalized cell lysate from experiment in panel c was quantified for intracellular Aβ40. The results were plotted as treatment concentration versus intracellular Aβ40 calculated relative to percent control (1% DMSO). In panels a−e, the wells treated with 1% DMSO alone served as control (100% Aβ), shown Aβ values are means ± SD with $n = 3$, and the result is representative of three separate experiments.

SB225002 Does Not Change α- and β-Secretase Activity. To determine whether the Aβ-lowering effect of SB225002 could be due to an increase in α-secretase activity (*15*) (thus competing with β- and γ-secretase mediated pathways), we treated 7w cells with different doses of SB225002 for 18 h. As controls, we used the

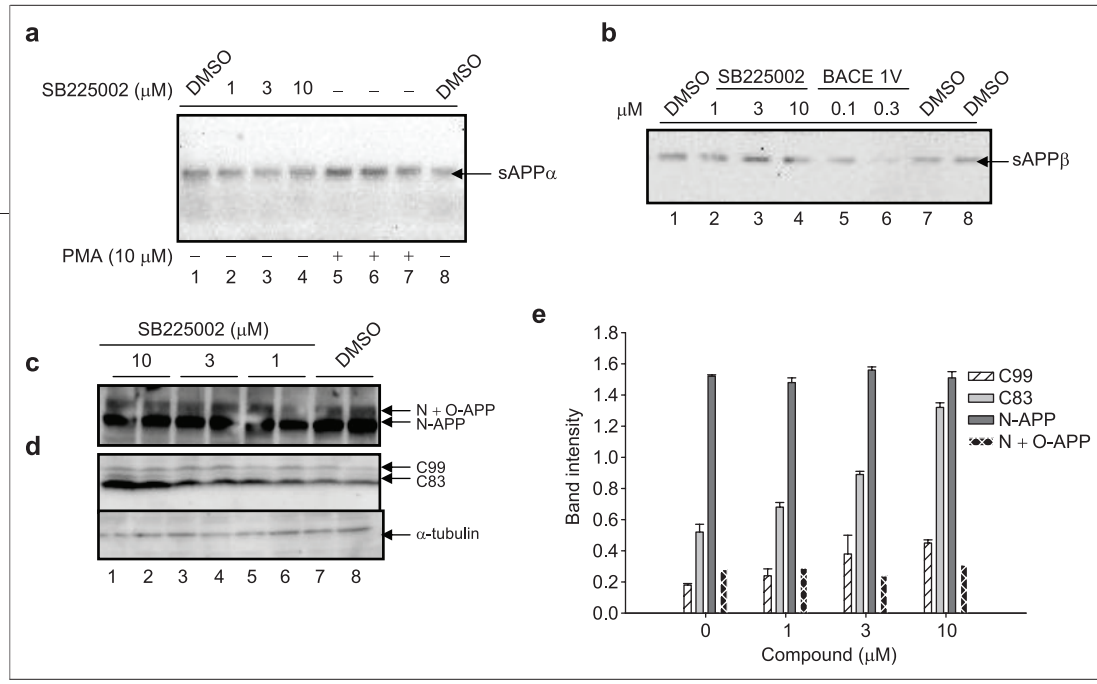

Figure 2. Effect of SB225002 on sAPPα (α-secretase product), sAPPβ (β-secretase product), C99 and C83 (substrates of γ-secretase), and APP. a) sAPPα was Western blotted (anti-APP N-terminus) from the medium of 7w cells after treatment with the indicated concentrations of SB225002 (lanes 2–4) or PMA (lanes 5–7). Lanes 1 and 8 were treated with 1% DMSO alone. b) sAPPβ was Western blotted (polyclonal sAPPβ antibody) from 7w cells media treated with the indicated concentrations of SB225002 (lanes 2–4), or BACE IV inhibitor (lanes 5 and 6). Lanes 1, 7, and 8 were treated with 1% DMSO alone. c) N- (lower band) and (N+O)-glycosylated (upper band) APP and d) C99 (upper band) and C83 (lower band) were Western blotted (anti-amyloid precursor protein C-terminal antibody) from protein-normalized 7w cell lysate treated with indicated doses of SB225002 (lanes 1–6). Lanes 7 and 8 were treated with 1% DMSO alone. Levels of α-tubulin were used as equal loading control. The result is representative of three separate experiments. e) Mean ± SD of the intensities of the N+O-APP, N-APP, C99 and C83 bands from three similar experiments.

known α-secretase activator phorbol-12-myristate-13-acetate (PMA, 10 μM) (*16*) and DMSO. As shown in Figure 2, panel a, SB225002 produces no change in levels of secreted soluble APPα (sAPPα, the soluble product of APP after α-secretase cleavage), whereas it was increased with PMA, suggesting that inhibition of Aβ by SB225002 is not a result of an increase in the activity of α-secretase. Since β-secretase is a rate-limiting enzyme in Aβ production, we also assessed the effect of SB225002 on the activity of β-secretase using 7w cells. The known β-secretase inhibitor BACE IV (*17*) was used as a control. As shown in Figure 2, panel b, no inhibition in levels of secreted soluble APPβ (sAPPβ, a soluble product from cleavage of APP by β-secretase) was found with SB225002 after treatment for 18 h. The unaltered sAPPβ and sAPPα levels suggest that inhibition of Aβ production with SB225002 is not mediated through either α- or β-secretases.

SB225002 Does Not Change the Level or Maturation of APP. Since altered APP holoprotein expression, maturation, or trafficking to the membrane can also affect production of Aβ, we next examined levels of APP after treatment of 7w cells with different concentrations of SB225002 for 18 h. Figure 2, panel c demonstrates that levels of both N- (lower band) and N- and O-glycosylated APP (upper band) essentially remained the same compared to control. This therefore ruled out changes in the expression level or maturation of APP as the reason for the observed dose-dependent reduction of Aβ by SB225002.

SB225002 Inhibits γ-Secretase Activity. We next evaluated SB225002 for its effect on the activity of γ-secretase, the enzyme involved in the final step of Aβ production. For this, we measured levels of C99 and C83, substrates for γ-secretase, after treatment with varying concentrations of SB225002 in 7w cells. As illustrated in Figure 2, panel d and quantified in Figure 2, panel e, treatment with SB225002 accumulated both C99 and C83 in a dose-dependent manner in 18 h, suggesting SB225002 inhibits Aβ production at the γ-secretase level. However, lack of inhibitory effect on Aβ levels at earlier time points at 4 and 6 h from our earlier observation indicates that the compound might not be a direct active-site inhibitor of γ-secretase.

SB225002 Down-Regulates PS. As inhibition of Aβ was affected by SB225002 after 18 h of treatment, we next asked if SB225002 indirectly reduces activity of γ-secretase through change in the expression levels of its four essential components, PS, Pen-2, Aph-1, and NCT. For this, we used the S1 cell line (Chinese hamster ovary cell line coexpressing all four components of γ-secretase; human PS1, Pen2-Flag, Aph1α2-HA, and

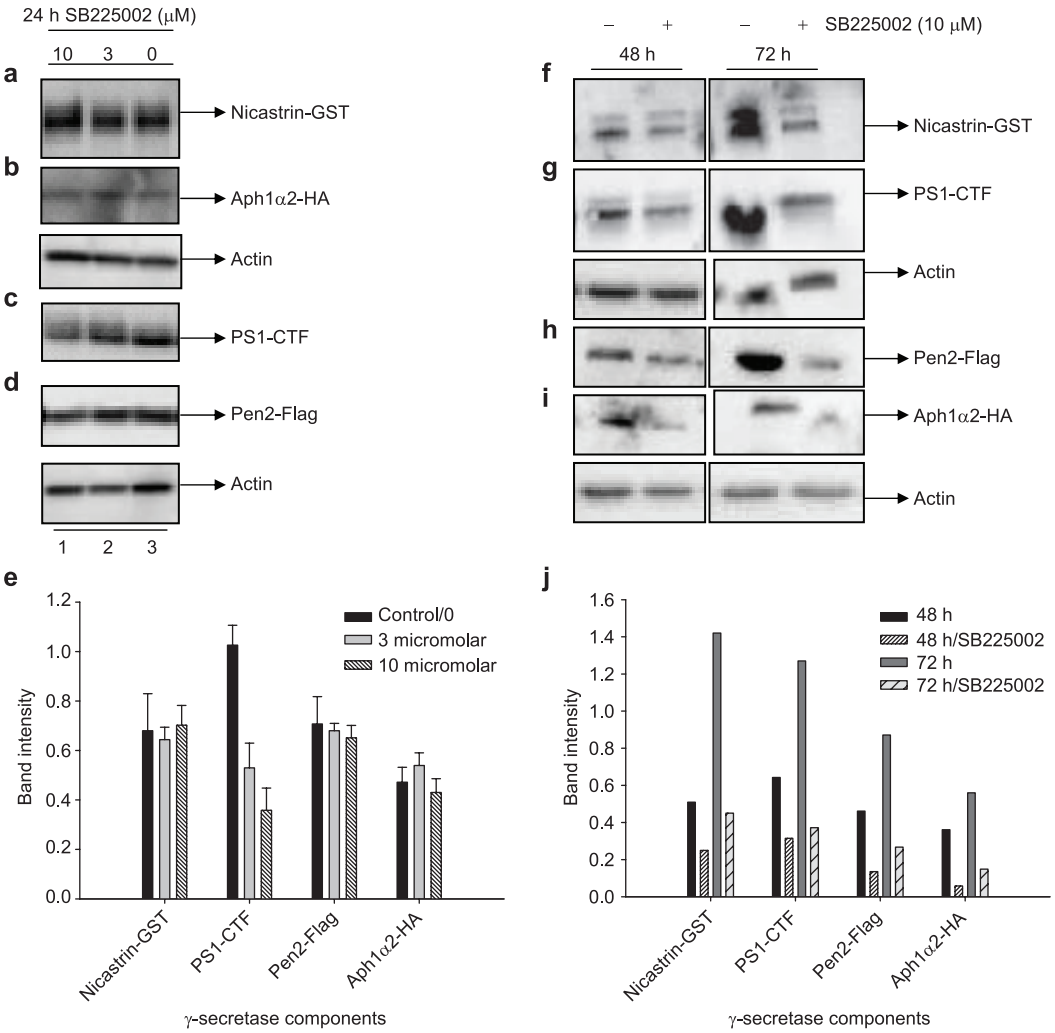

Figure 3. Effect of SB225002 on components of γ-secretase in S1 CHO cells (stably coexpressing human PS1, Flag-Pen-2, Aph1α2-HA, and NCT-GST). a–e) Protein-normalized cell lysate from S1 cells treated with SB225002 at indicated concentrations was Western blotted for (panel a) NCT-GST (anti-GST), (panel b) Aph1α2-HA (3F10), (panel c) PS (anti-CTF Presenilin), and (panel d) Pen2-Flag (anti-Flag M2 monoclonal antibody). e) Mean ± SD of the intensities of the NCT-GST, PS1-CTF, Pen2-Flag and Aph1α2-HA bands from four similar experiments. Paired *t* test indicated a significant reduction ($p < 0.01$) of the PS1-CTF level in the treatment cultures relative to the control cultures. f–j) Protein-normalized cell lysate from S1 cells treated with SB225002 at indicated concentration for 48 and 72 h was Western blotted for (panel f) NCT-GST (anti-GST), (panel g) PS (anti-CTF Presenilin), (panel h) Pen2-Flag (anti-Flag M2 monoclonal antibody), and (panel i) Aph1α2-HA (3F10). j) Intensities of the NCT-GST, PS1-CTF, Pen2-Flag, and Aph1α2-HA bands.

NCT-GST) (*18*). The antagonist SB225002 was tested for its effect on NCT-GST, PS1-CTF, Pen2-Flag, and Aph1α2-HA (Figure 3, panels a–d) at two different concentrations (3 and 10 μM), along with 1% DMSO (0) as a control. The quantification of bands for components of γ-secretase (Figure 3, panel e) demonstrated significant dose-dependent reduction ($p < 0.01$) of the PS1-CTF levels in the treatment cultures relative to the control cultures, suggesting that effect of SB225002 on γ-secretase activity is mediated *via* change in PS level. The down-regulation of PS was further verified using quantitative real-time PCR of treated cells and was found

to be 1.8−2-fold less than control (data not shown). However, other components of γ-secretase did not show change at the transcription level. Further, on increasing the incubation treatment time to 48 and 72 h, we observed reduction in levels of other components (Figure 3, panels f−j) consistent with previous findings suggesting coordinated regulation among γ-secretase components at the protein level (*19*–*23*).

SB225002 Prevents Processing of Notch*ΔE* (N*ΔE*). As γ-secretase is known to affect proteolysis of numerous type I membrane proteins, we next asked if SB225002 inhibits γ-secretase independently of substrate selectivity. To ascertain the effect of SB225002 on Notch cleavage, we treated N7 cells (*24*) (HEK cell line overexpressing Notch*ΔE*). We found that the processing of N*ΔE* by γ-secretase was reduced in a dose-dependent manner (Figure 4, panel a) similar to that of DAPT, a direct inhibitor of γ-secretase as compared to control. Consistent with the mediating effect of CXCR2 on cleavage of C99 and C83 in 7w and HEKsw cells, these results suggest that CXCR2 can mediate γ-secretase cleavage of its other substrates as well.

SB225002 Inhibits AICD Production. Recent evidence from mutation studies of PS and processing of APP suggest the possibility of inhibition of Aβ (γ-cleavage) without affecting AICD (ε-cleavage; 49−99/50−99 fragments) (*25*). Therefore, we next investigated the influence of SB225002, if any, on the inhibition of AICD. The ability of SB225002 to affect AICD was evaluated using a previously developed TREX293 inducible Luciferase reporter cell-based screening method (*26*). The outcome in this assay is liberation of AICD, along with the fused GV transcription factor (AICD-GV) generated as a consequence of either α/γ or β/γ secretase activity. To determine the effect on AICD release, different concentrations of SB225002 and DAPT were used to treat the inducible reporter system after the addition of a fixed concentration of tetracycline. SB225002 inhibited the luciferase signal from AICD-GV in a dose-dependent manner in three separate experiments with an IC_{50} of 1−3 μM (Figure 4, panel b) after 18 h of incubation. The IC_{50} for inhibition of AICD for both compounds (SB225002 and DAPT) was found to be higher than that for inhibition of Aβ40 and Aβ42 measured in the 7w cells, but these IC_{50}'s were similar to the IC_{50} in the HEKsw cells. These differences might be due to intrinsic cell line differences in metabolism of APP or expression levels of exogenous APP. Since we established

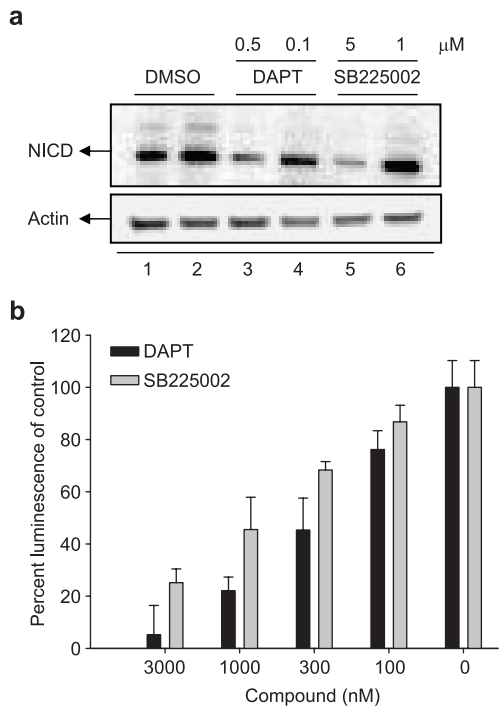

Figure 4. Effect of SB225002 on the Notch and APP intracellular domain (AICD). a) Protein-normalized cell lysate from Notch*ΔE* cells treated with SB225002 and DAPT at indicated concentrations along with DMSO was Western blotted for NICD. b) Both compounds SB225002 and control DAPT at the indicated concentrations were incubated with the TREX 293 (HEK293) cell line. The observed luminescence (due to released AICD-GV domain) was corrected for protein variation and basal luminescence and calculated as percentage relative to luminescence in control cultures (where control cultures is cells treated with 1% DMSO and represents 100% luminescence). Error bars indicate standard deviation from the mean of the luminescence values with *n* = 8. The result is representative of three separate experiments.

earlier that SB225002 does not affect α- or β-secretases, inhibition of luminescence in this assay can be explained as the inhibition of both γ- and ε-cleavage by SB225002. Inhibition of AICD-GV, Aβ40, and Aβ42 along with accumulation of γ-secretase substrates further supports the notion that SB225002 is inhibiting γ-secretase activity (*27*).

SB225002 Lowers Aβ40 Production in a Transgenic Mouse Model. To investigate whether the observed reduction in Aβ40 and Aβ42 caused by SB225002 was relevant to *in vivo* conditions, we employed the trans-

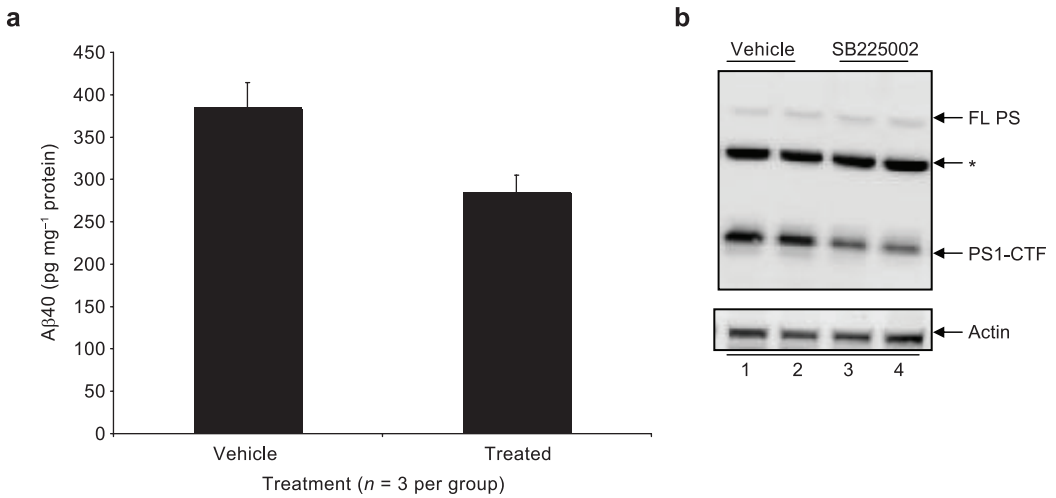

Figure 5. Effect of acute SB225002 treatment on soluble Aβ40 and PS levels in the brain of PS (M146L) APPsw transgenic mice. a) After protein normalization, Aβ40 levels (mean ± SD with $n = 3$) from cortex of PS (M146L) APPsw treated either with vehicle or SB225002 were determined using ELISA. The result is representative of two separate ELISAs. Paired *t* test performed showed a significant reduction ($p < 0.01$) of the soluble Aβ40 brain levels in the treated group animals relative to the control group. b) Protein-normalized tissue lysate from experiment in panel a was Western blotted for PS. Asterisk (*) denotes nonspecific band.

genic PS (M146L) APPsw (PSAPP) (*28*) mouse model of cerebral amyloidosis. Eleven-month-old PSAPP mice were treated subcutaneously for 3 consecutive days, once daily, with either vehicle (100 μL of saline containing 1% DMSO and 20% of β-cyclodextrin (w/v); $n = 3$) or SB225002 (0.2 mg kg^{-1} day^{-1}; $n = 3$) in vehicle and euthanized on day 3.

In this preliminary acute treatment study, soluble levels of Aβ40 were significantly reduced ($p < 0.01$) by ~25−30% (Figure 5, panel a) in the brains of PSAPP mice receiving treatment with the CXCR2 inhibitor as compared to control animals. No obvious signs of behavioral abnormalities were observed for any of the treated animals at the indicated dose. On analysis of brain tissue lysate, we found reduction in levels of PS1-CTF with no increase in full length PS (FLPS) level (Figure 5, panel b). Our previous finding of reduction in PS expression (using quantitated rt-PCR) with no change in FLPS at the protein level and reduction in endoproteolysed products of PS suggests a negative feedback control. This hypothesis is consistent with recent findings by two independent laboratories that demonstrate that inhibition of basal activity of c-jun-NH2-terminal kinase (JNK) represses the expression of presenilin-1 (*29*) maybe through reduced stabilization of PS1-CTF (*30*). It is plausible but speculative that CXCR2 might mediate stabilization of PS1-CTF (half-life ~24 h) (*31*), an endoproteolyzed product of PS. The inhibition of CXCR2 may reduce the stability of PS1-CTF that in turn inhibits endoproteolysis of FLPS (half-life ~1 h) (*31*) which then negatively regulates its expression. Nevertheless, whatever the precise biochemical mechanism for repression in PS expression, our findings are pharmacologically relevant and could have major therapeutic implications.

Repertaxin, an Allosteric Inhibitor of CXCR2 Inhibits Aβ40 Generation. Next, we asked whether the decrease in Aβ was a result of a nonselective effect of the compound SB225002 or a specific consequence of CXCR2 receptor antagonism. To determine this, we selected Repertaxin (*32*), an allosteric inhibitor (*31*) of the CXCR2 receptor that is chemically unrelated to SB225002. Interestingly, Repertaxin after 18 h of treatment also showed a dose-dependent reduction of Aβ (Figure 6) with an IC_{50} of ~5 μM, almost 10-fold higher than that of SB225002, in 7w cells. The reduction in Aβ with two chemically unrelated antagonists SB225002 and Repertaxin suggests that CXCR2 antagonism might alter APP processing. Since SB225002 and Repertaxin bind to the CXCR2 receptor at different sites, this suggests that their combined use might synergistically increase Aβ in-

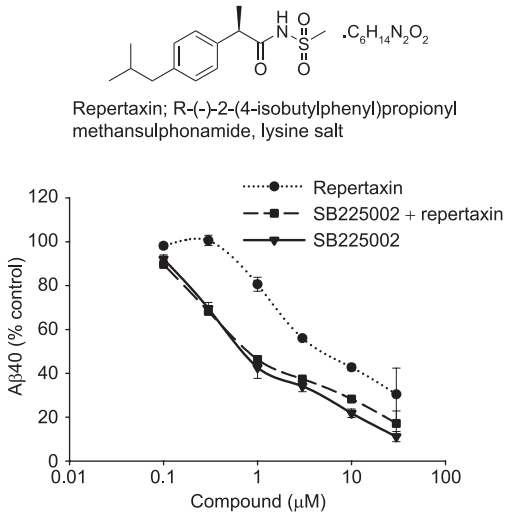

Figure 6. Effect of Repertaxin, an allosteric antagonist of CXCR2, alone and in combination with SB225002 on Aβ40. 7w cells were treated with Repertaxin alone and a combination of Repertaxin and SB225002. Aβ40 measured by ELISA was calculated relative to percent control and plotted versus concentration as mean ± SD with $n = 3$. Aβ40 measured from SB225002 treatment alone was plotted for comparison. The result is representative of three separate experiments.

hibition. To test this, we treated 7w cells with SB225002 alone and combinations of SB225002 and Repertaxin. As illustrated in Figure 6, SB225002 and Repertaxin together did not amplify the inhibition of Aβ40 production. The lack of synergy between SB225002 and Repertaxin might be because of higher binding affinity of SB225002 for the IL8 binding site (*33*) compared to Repertaxin at the allosteric site. The binding of SB225002 might also be changing the conformation of CXCR2, thus preventing binding of Repertaxin to its allosteric site. Since both Repertaxin and SB225002 bear some structural resemblance (*34*) with known glycogen synthase kinase-3β (GSK3β) inhibitors (that are known to affect γ-secretase activity through phosphorylation of the hydrophilic loop of PS1), we next asked if SB225002 and Repertaxin inhibit GSK3β. To ascertain GSK3β inhibition by CXCR2 antagonists, we treated HEKsw cells with these compounds at 5 μM concentration along with 1% DMSO as control using a previously described procedure (*35*) (Supporting Information). The compounds did not show inhibition of GSK3β activity (Supporting Information), suggesting that inhibition of γ-secretase by these compounds is not through GSK3β.

Stimulation of CXCR2 Receptor Enhances Aβ Production. The fact that application of Repertaxin (*32*), another antagonist of CXCR2, also inhibited Aβ suggested a possible general influence of CXCR2 on APP processing. To further explore function of CXCR2 in regulation of Aβ production, we studied CXCR2's natural ligands, IL8 and GROα. The chemokines IL8 and GRO-α bind and activate CXCR2, which then undergoes endocytosis and plays a role in intracellular signaling (*36, 37*). For activation of CXCR2, we treated 7w cells in the presence of 1 μg mL^{-1} of human recombinant proteins IL8 (hrIL8) or hrGRO-α. We selected this particular concentration on the basis of earlier studies done on the activation and endocytosis of CXCR2 (*36*). As illustrated in Figure 7, panel a (quantitative analysis using ELISA) and Figure 7, panel b, both hrIL8 and hrGRO-α treatment significantly increased ($p < 0.001$) levels of Aβ40 relative to those of control cultures after 24 h. However no significant change in Aβ40 was observed at 4 h (Figure 7, panel a). Following 24 h of treatment with these chemokines, analysis of cell lysate showed no change in CXCR2 expression level (Figure 7, panel c). However, an increase in PS1-CTF level was noted (Figure 7, panel d). This is consistent with our earlier finding of the SB225002 effect on reducing levels of PS. This substantial increase in PS level in the presence of CXCR2 agonists implicates CXCR2 as a mediator (when interacting with chemokine) in regulating PS level.

As addition of hrIL8 and hrGRO-α significantly affected baseline production of Aβ, we next asked if neutralization of endogenous ligands in cell culture by their antibodies would have any influence on Aβ production. To this end, we tested three concentrations (0.1, 0.3, and 1 μg mL^{-1}) of antibodies for IL8, GRO-α, and MIP-1β (another chemokine) for their effect on the production of Aβ in the 7w cell line at two time points, 4 and 24 h. Interestingly, neutralization with antibodies showed a significant reduction in levels of Aβ40 between treated and control cultures at higher concentrations in 4 h (anti-Groα, 1 μg mL^{-1}, $p < 0.001$; anti-IL8, 0.3 μg mL^{-1}, $p < 0.01$; anti-IL8, 1 μg mL^{-1}, $p < 0.001$) (Figure 7, panel e). In 24 h a significant dose-dependent reduction in Aβ40 levels was observed with anti-MIP-1β and anti-IL8 treatment ($p < 0.001$), whereas reduction in Aβ40 was significant ($p < 0.001$) but not dose-

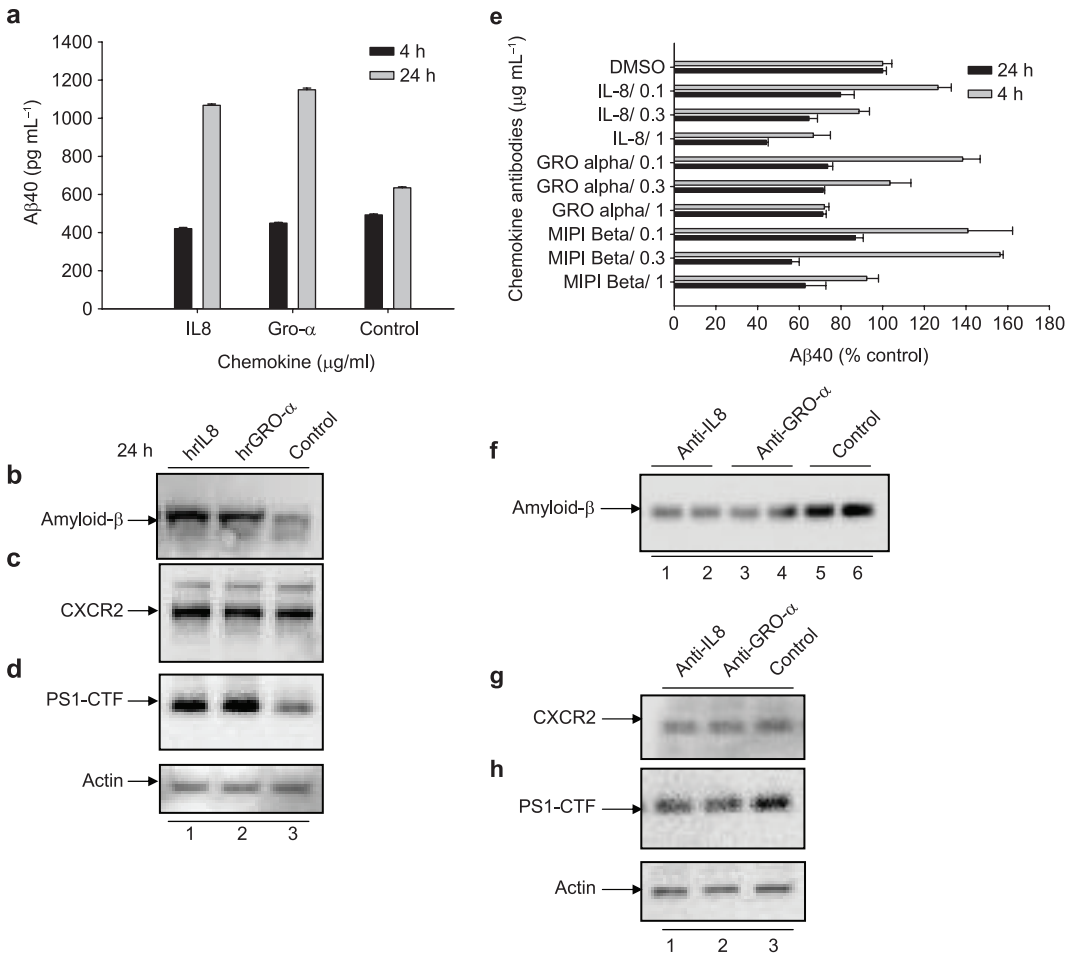

Figure 7. Effect of chemokines treatment or immunodepletion on Aβ production and PS-CTF levels. a) Following incubation of 7w cells for 4 and 24 h, with hrGROα or hrIL8 at 1 μg mL^{-1}, protein-normalized media was quantified for Aβ40 calculated relative to percent control cultures and plotted as mean ± SD with $n = 3$. The result is representative of three separate experiments. Paired *t* test indicated a significant increase ($p < 0.001$) of the Aβ40 levels in the CXCR2 agonist treated cultures relative to the control cultures. b) Media from 7w cells incubated for 24 h with hrIL8 (1 μg mL^{-1}), hrGRO-α (1 μg mL^{-1}), or control was Western blotted with 6E10 antibody for detection of Aβ. c,d) Protein-normalized cell lysate, obtained from experiment in panel b was Western blotted for (panel c) CXCR2 and (panel d) PS. Levels of Actin served as equal loading controls. e) Different doses of antibodies for IL8, GROα and MIP-1β were incubated with 7w cells at two time points, 4 and 24 h. Media collected at those time points was quantified for Aβ40 as in panel a and plotted as mean ± SD with $n = 3$. The result is representative of three separate experiments. Paired *t* test indicated a significant increase at certain low concentration of antibodies (anti-MIP-1β, 0.1 and 0.3 μg mL^{-1}, $p < 0.001$; anti-Groα, 0.1 μg mL^{-1}, $p < 0.001$; anti-IL8, 0.1 μg mL^{-1}, $p < 0.001$) of the Aβ40 levels in the treated cultures relative to the control cultures for 4 h. However, significant reduction in levels of Aβ40 between treated and control cultures was observed at higher concentrations in 4 h (anti-Groα, 1 μg mL^{-1}, $p < 0.001$; anti-IL8, 0.3 μg mL^{-1}, $p < 0.01$; anti-IL8, 1 μg mL^{-1}, $p < 0.001$). In 24 h significant dose-dependent reduction in Aβ40 levels was observed with anti-MIP-1β and anti-IL8 treatment ($p < 0.001$), whereas reduction in Aβ40 was significant ($p < 0.001$) but not dose-dependent in the anti-Groα treatment group. f) Protein-normalized media from 7w cells incubated for 24 h with anti-IL8 (1 μg mL^{-1}) and anti-GRO-α (1 μg mL^{-1}) and control was Western blotted with 6E10 antibody for detection of Aβ. g,h) Protein-normalized cell lysate obtained from experiment panel f was Western blotted for (panel g) CXCR2 and (panel h) PS. Levels of Actin served as equal loading controls.

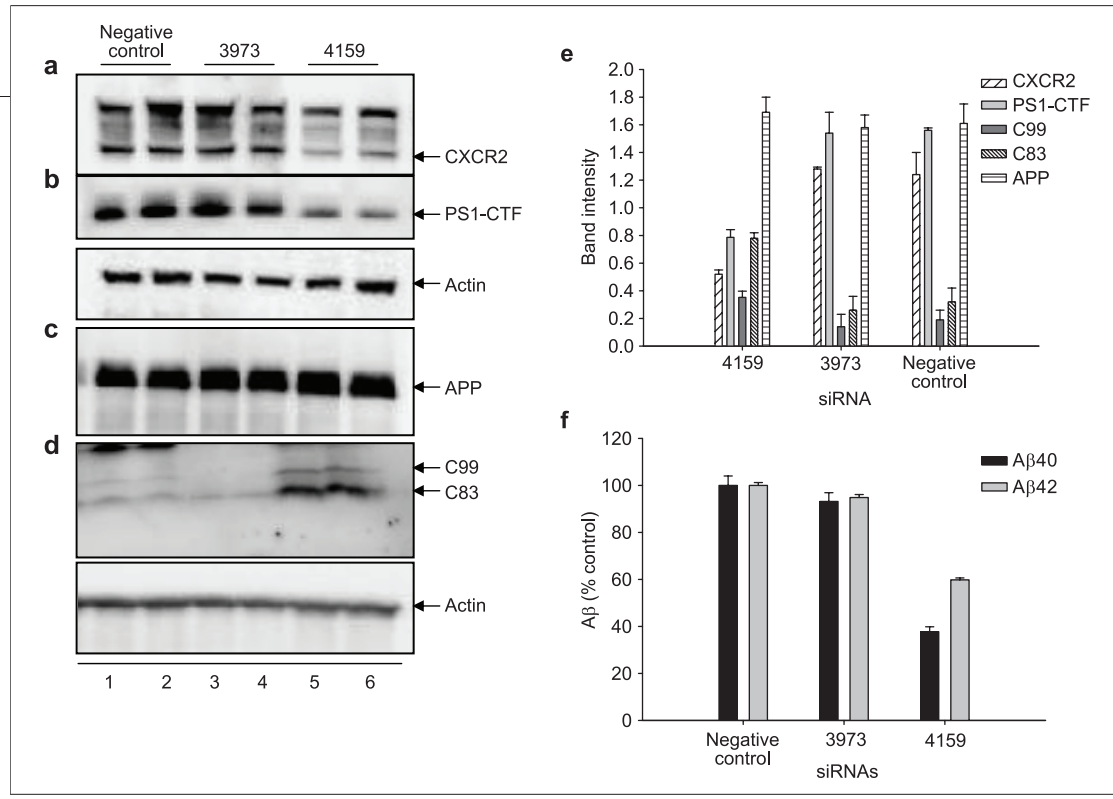

Figure 8. Effect of knockdown of CXCR2 on APP, C99, C83, Aβ40, and Aβ42. a–d) CXCR2 from the HEKsw cell line was knocked down using the two indicated small interfering RNAs (siRNAs) and a scrambled sequence siRNA negative control. After 72 h, protein-normalized cell lysates were Western blotted for (panel a) CXCR2 (anti-CXCR2), (panel b) PS (anti-CTF Presenilin), (panel c) APP (APP-CTF antibody), and (panel d) C99 (upper band) and C83 (lower band) (APP-CTF antibody). Levels of Actin served as equal loading controls. e) Mean ± SD of the intensities of the CXCR2, APP, C99, and C83 from three similar experiments. Paired *t* test indicated a significant increase (4159, $p < 0.001$) of the C99 and C83 and slight increase in FL APP (4159, $p < 0.01$) band intensities in the CXCR2 knocked-down cultures relative to the treatment cultures. f) The effect on Aβ40 and on Aβ42 was quantified from protein-normalized media collected from HEKsw cells transfected with the indicated siRNAs (calculated relative to percent negative control as means ± SD with $n = 3$). The result is representative of three separate experiments. Paired *t* test indicated a significant reduction (4159, $p < 0.001$) in the Aβ40 and Aβ42 levels in the CXCR2 knocked down cultures relative to the control cultures.

dependent in the anti-GROα treatment group (the inhibition by this antibody might plateau at these doses) (Figure 7, panel e). Also, significant increases at certain low concentrations of antibodies (anti-MIP-1β, 0.1 and 0.3 μg mL^{-1}, $p < 0.001$; anti-Groα, 0.1 μg mL^{-1}, $p < 0.001$; anti-IL8, 0.1 μg mL^{-1}, $p < 0.001$) of the Aβ40 levels in the treated cultures relative to those of the control cultures for 4 h was noted (Figure 7, panel e). Figure 7, panel f shows change in Aβ 24 h after treatment with 1 μg mL^{-1} of anti-IL8 or anti-GRO-α in 7w cells. Analysis of the cell lysate after 24 h of treatment showed no change in CXCR2 level (Figure 7, panel g). However, the level of PS1-CTF was reduced in treated cells versus control (Figure 7, panel h). The change in PS level and subsequent altered Aβ levels, with addition of chemokines or immunodepletion of endogenous chemokines, further implicates CXCR2 involvement in APP processing.

CXCR2 Receptor Knockdown Reduces Aβ40 and Aβ42 with Accumulation of C99 and C83. To further understand and establish CXCR2's role in processing of APP and production of Aβ, we evaluated changes in levels of APP, C99 and C83, Aβ40, and Aβ42 after down-regulation of CXCR2. The knockdown of human CXCR2 expressed in the HEKsw cell line was examined using three small interfering RNAs (siRNA). To determine the extent of knockdown, a siRNA negative control (scrambled sequence siRNA) was used. No cell toxicity was observed as determined by the lactate dehydrogenase (LDH) Cyto-tox kit, but a decrease in cell proliferation (determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay) was found with siRNA 4067. Because of reduction in cell proliferation on treatment with 4067 siRNA, it was not considered for further studies. As shown in Figure 8, panel a, we were able to achieve significant knockdown of CX-

CR2 in 72 h with siRNA 4159 and almost no reduction with siRNA 3973. Consistent with our previous finding using CXCR2 antagonists, we noted reduction in PS1-CTF levels (Figure 8, panel b). Next, to see whether knockdown of CXCR2 had any effect on the expression of APP, the membrane was stripped and probed with an APP-CTF antibody. As illustrated in Figure 8, panel c, no decrease in levels of APP was observed (instead an increase in APP (Figure 8, panel c) of 4159, $p < 0.01$ was observed), and interestingly, significant accumulation (Figure 8, panel d; 4159, $p < 0.001$) of C99 and C83 (Figure 8, panels d and e) was noted, suggesting inhibition of γ-secretase in the absence of CXCR2. Quantitative analysis of Aβ40 and Aβ42 (Figure 8, panel f) in media collected from CXCR2 knockdown cells using ELISA showed significant reduction of both Aβ40 and Aβ42 with siRNA 4159 ($p < 0.001$) consistent with the degree of CXCR2 knockdown. This further strengthens the role of CXCR2 in regulation of APP processing.

Conclusions. In the present study, we explored the role of the chemokine receptor CXCR2 in relation to Aβ production. Screening of a focused library of antagonists of chemokine receptors identified a selective ligand of CXCR2, SB225002 (*10*). The compound potently inhibited Aβ production *via* inhibition of γ-secretase, leading to the accumulation of APP fragments C99 and C83. Additionally, we found inhibition of AICD by SB225002, further suggesting that SB225002 impacts γ-secretase because (i) reduction of AICD by inhibition of β-secretase would not be detectable in this assay that utilizes TREX 293 (HEK 293) cells, which have high endogenous α- and γ-secretase activity (*26*), and (ii) reduction of Aβ by stimulation of the α-secretase pathway would not decrease luciferase activity. Also the observed inhibition of Aβ and AICD by SB225002 is in agreement with previous findings (*27, 38*) that the same PS/γ-secretase is responsible for both γ- and ε-cleavages of APP. Furthermore, the inhibitory effect of this compound on γ-secretase was found to be mediated through a reduction in the PS1-CTF level. Importantly, SB225002 demonstrated *in vivo* efficacy in a 3-day acute study with ~25−30% reduction in soluble Aβ40 in the brain of PSAPP (*28*) mice. Further, Repertaxin (*32*), an allosteric inhibitor of CXCR2, demonstrated that general inhibition of CXCR2 is effective in blocking Aβ production. Consistently, we found that stimulation of CXCR2 with hrIL8 and hrGRO-α, both ligands of CXCR2, significantly increased Aβ production with increases in the PS1-CTF level, further implicating a role for CXCR2 as a mediator of APP processing. In addition, the immunodepletion of endogenous CXCR2 ligands with their respective antibodies resulted in reduction of Aβ.

To further verify the role of CXCR2 on γ-secretase activity, we transiently knocked-down CXCR2 with siRNA. We noted significant accumulation of C99 and C83 with reduction in levels of PS1-CTF and of both Aβ40 and Aβ42 again, implying modulation of Aβ levels *via* changes in γ-secretase activity. Also, since the use of an antibody against either IL8 or GRO-α results in the reduction of PS1-CTF but not CXCR2 in these cells, this suggests that the regulation of PS levels is downstream of CXCR2 signaling. Taken together, we propose that CXCR2 ligation with IL8 and GRO-α can enhance APP proteolysis through an increase in PS level. Further studies using CXCR2 knockout mice crossed with PSAPP, pathological analysis, and detailed SAR of SB225002 are currently underway.

Our present study supports that secretase-mediated proteolysis of APP can be subject to multiple levels of regulation by intracellular pathways, providing a coordinated proteolysis of APP for the stringent production of Aβ in physiological conditions (*24*). Given the upregulation of CXCR2 in the AD brain, the demonstration here that its knockdown *in vitro* can reduce Aβ levels, and its tractability for small molecule antagonism, we propose that CXCR2 may provide an additional important therapeutic target for AD.

METHODS

Cell Lines and Cultures. All cells were cultured at 37 °C in the presence of 5% CO_2. The CHO wt cell line was purchased from ATCC and was maintained as per instructions. The 7w cells (CHO cell line stably expressing wtAPP751), HEKsw cells (human embryonic kidney cell line stably expressing APP with Swedish mutation), the S-1 cells (stable CHO cell line coexpressing human PS1, FLAG-Pen-2, Aph1α2-HA, and NCT-GST), TREX 293 HEK cell line (expressing APP695 fused with Gal4VP16 at its C-terminus), 2A2 cell line (stable CHO cell line coexpressing wt APP, D257A PS1, and D366A PS2), and N7 cell line (stable HEK cell line expressing NotchΔE) were cultured as described previously (*11, 18, 24, 26, 39*).

CXCR2 Knockdown with Interfering RNA. HEKsw cells plated onto a six-well plate were treated with three predesigned siRNAs (4159, 4067, and 3973) and negative control purchased

from Ambion. Delivery of siRNA was performed using Lipofectamine 2000 complexed with 120 pmol of siRNA. At different time points starting from 64, 72, 96, and 120 h, media was collected and quantified for Aβ40 and Aβ42 from protein normalized conditioned media as described earlier and cells were lysed. Equivalent amounts of proteins were resolved by Nu-PAGE gels and analyzed by Western blotting.

Quantitative Densitometry and Statistical Analysis. The relative density of immunoreactive bands was determined using MCID analysis 7.0 (Imaging Research Inc.) and quantitative analysis was performed using Sigma plot 8.0. Results were expressed as the mean ± SD. All dose response graphs were plotted using Sigma plot 8.0 and the standard sigmoid 4-parameter equation

$$y = y_0 + \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}}$$

was used to fit the dose−response data. The scaling of the *x*-axis is in log. Data were compared using a paired *t* test analysis. Statistical significance was defined as follows: $p < 0.05$, $p < 0.01$, and $p < 0.001$.

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Supporting Information Available: This material is available free of charge *via* the Internet.

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