Afbazobole Activation of $\sigma$-1 Receptors Modulates Neuronal Responses to Amyloid-$\beta$_{25–35}

Adam A. Behensky, Ilya E. Yasny, Alexander M. Shuster, Sergei B. Seredenin, Andrey V. Petrov, and Javier Cuevas

Department of Molecular Pharmacology and Physiology, University of South Florida, College of Medicine, Tampa, Florida (A.A.B., J.C.); IBC Generium, Volginsky, Russian Federation (I.E.Y., A.M.S., A.V.P.); and Zakusov Institute of Pharmacology, Russian Academy of Medical Sciences, Moscow, Russian Federation (S.B.S.)

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ABSTRACT

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by a continual decline of cognitive function. No therapy has been identified that can effectively halt or reverse its progression. One hallmark of AD is accumulation of the amyloid-$\beta$ peptide (A$\beta$), which alone induces neuronal injury via various mechanisms. Data presented here demonstrate that prolonged exposure (1–24 hours) of rat cortical neurons to A$\beta$_{25–35} results in an increase in basal intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$), and that coincubation with the compound afobazole inhibits these [Ca$^{2+}$]$_i$ increases. The effect of afobazole on [Ca$^{2+}$]$_i$ is due to activation of $\sigma$-1 receptors but could not be mimicked by a second pan-selective $\sigma$ receptor agonist, 1,3-di-o-tolyguanidine (DTG). Afbazobole was also found to lessen nitric oxide (NO) production in response to A$\beta$_{25–35} application but did not affect elevations in reactive oxygen species elicited by the A$\beta$ fragment. The reductions in [Ca$^{2+}$]$_i$ and NO perturbation produced by afobazole were associated with a decrease in neuronal cell death, whereas DTG failed to enhance cell survival. Examining the molecular mechanisms involved in the increased neuronal survival demonstrates that afobazole incubation results in lower expression of the proapoptotic protein Bax and the death protease caspase-3, while at the same time increasing expression of the antiapoptotic protein, Bcl-2. Given the importance of A$\beta$ neurotoxicity in AD etiology, the findings reported here suggest that afobazole may be an effective AD therapeutic agent. Furthermore, $\sigma$-1 receptors may represent a useful target for AD treatment, although not all $\sigma$-1 ligands appear to be equally beneficial.

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder that is the most common form of senile dementia in the United States (Gaugler et al., 2013). Although the full pathophysiological underpinnings of AD remain elusive, one of the hallmarks of AD is an increase in amyloid-$\beta$ (A$\beta$) plaque formation in the brain (Selkoe, 2001). The A$\beta$ fragment A$\beta$_{25–35}, which has been found in AD brains, has been proposed as a major contributor to the pathogenesis of AD (Ramkissoon et al., 2010). This A$\beta$ fragment has been shown to induce injury via various mechanisms, including production of reactive oxygen species (ROS) (Schubert et al., 1995), dysregulation in intracellular Ca$^{2+}$ (Joseph and Han, 1992), enhanced N-methyl-D-aspartate (NMDA) channel activity (Molnar et al., 2004), and increased activation of caspase-3 (Marin et al., 2000).

Previous studies have shown that a possible link might exist between $\sigma$ receptors and the etiology of AD. A loss of $\sigma$ binding sites has been observed in AD, and specific $\sigma$-1 receptor polymorphisms have been associated with altered risk for this disease (Jansen et al., 1993, Uchida et al., 2005, Mishina et al., 2008, Feher et al., 2012). Moreover, $\sigma$-1-selective agonists have been shown to reduce memory loss produced by A$\beta$_{25–35} injections in mice (Maurice et al., 1998). Recently, tetrahydro-N,N-dimethyl-2', 2-diphenyl-3-furanmethanamine hydrochloride (ANAVEX2-73) was shown to reduce $\tau$ hyperphosphorylation and A$\beta$_{1–42} generation in a mouse model of Alzheimer’s disease via the activation of both $\sigma$-1 and muscarinic receptors (Lahmy et al., 2013). However, it is unclear whether other $\sigma$-receptor ligands, particularly ligands that do not affect muscarinic receptors, have similar neuroprotective properties. However, the molecular mechanism(s) by which $\sigma$-1 receptors decrease neuronal injury upon A$\beta$ exposure have not been identified, and the effects of $\sigma$-2-receptor activation on neuronal responses to A$\beta$ need to be elucidated.

The $\sigma$ receptors, once thought to be a class of opioid receptors, are known to have widespread regulatory actions during neuropathophysiological states (Tsai et al., 2009). The $\sigma$-1 receptor has been shown to be an interorganelle chaperone
localized to the mitochondrion-associated endoplasmic reticulum membrane (Su et al., 2010). The α-1 receptor–mitochondrion-associated endoplasmic reticulum membrane relationship plays important roles in cell signaling, mitochondrial function, and maintaining intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) homeostasis (Su et al., 2010). Furthermore, upon stimulation with high agonist concentrations, α-1 receptors migrate to subplasmalemmal endoplasmic reticulum or the plasma membrane and interact with various ion channels (Su et al., 2010). In response to ischemia or acidosis, activation of α-1 receptors prevents [Ca\(^{2+}\)]\(_i\) overload and concomitant cell death, likely via actions at multiple targets, including acid-sensing ion channels and NMDA receptors (Katnik et al., 2006, Herrera et al., 2008). In addition to regulation of [Ca\(^{2+}\)]\(_i\), homeostasis, other α-receptor–mediated effects may contribute to neuroprotection, including decreased production of ROS and reactive nitrogen species, activation of the antiapoptotic protein Bcl-2, and inhibition of the proapoptotic protein Bax (Tcheder and Yorio, 2008, Meunier and Hayashi, 2010, Kourrich et al., 2012, Pal et al., 2012). It remains to be established if some or all of these effects are linked to α-receptor–mediated neuroprotection following neuronal ischemia, and if they contribute to neuroprotection following Aβ exposure.

Our laboratory recently showed that 5-ethoxy-2-[2-(morpholino)-ethylthio]benzimidazole (afobazole), a drug currently used in Russia to treat anxiety and panic disorders, is both a α-1- and α-2-receptor agonist and provides neuroprotection in an in vitro ischemia model (Cuevas et al., 2011a,b). Unlike ANAVEX2-73, afobazole does not interact with muscarinic receptors (Seredenin and Voronin, 2009). Activation of α-1 receptors by afobazole results in a decrease in ischemia-induced Ca\(^{2+}\) overload, which is due in part to inhibition of NMDA channel activation (Katnik et al., 2006, Cuevas et al., 2011a). Previous studies have suggested other mechanisms for the neuroprotective properties of afobazole, including decreased caspase-3 activation and reduced oxidative stress (Zenina et al., 2005, Antipova et al., 2010). Thus, afobazole is an excellent ligand for examining the relationship between α receptors and Aβ-induced neurotoxicity, and is a potential candidate for the treatment of Alzheimer’s disease.

Experiments were carried out to determine how afobazole affects neuronal responses to the Aβ fragment, Aβ\(_{25-35}\). Afobazole mitigated Aβ\(_{25-35}\)-evoked increases in [Ca\(^{2+}\)]\(_i\) in neurons, and these effects were blocked by inhibition of α-1 but not α-2 receptors. Afobazole was also found to lessen nitric oxide (NO) production in response to Aβ\(_{25-35}\) application and reduce neuronal death. The decrease in cell death produced by afobazole was associated with reduced neuronal expression of the proapoptotic protein Bax and the death protease caspase-3 and enhanced expression of the antiapoptotic gene product Bcl-2.

## Materials and Methods

### Preparation of Cortical Neurons

All experiments were carried out on cultured cortical neurons from mixed sex embryonic day 18 (E18) Sprague-Dawley rats (Harlan, Indianapolis, IN). Methods used here were identical to those previously reported for the isolation and culturing of these cells (Katnik et al., 2006). Cortical neurons were plated on poly(L-lysine) coated cover slips and cultured in B27- and L-glutamine–supplemented Neurobasal medium (Neurobasal Complete) (Life Technologies, Grand Island, NY). Cells were used for experiments after 10–21 days in culture, which permits synaptic contact formation and yields robust responses to other pathophysiologic conditions such as in vitro ischemia and acidosis (Katnik et al., 2006, Herrera et al., 2008). Animals were cared for in accordance with the regulations and guidelines set forth by the University of South Florida’s College of Medicine Institution on Animal Care and Use Committee.

### Calcium Imaging

Intracellular Ca\(^{2+}\) concentrations were measured in isolated cortical neurons using ratiometric fluorometry as previously described (Katnik et al., 2006). For most experiments, cultured neurons were incubated in 25 μM Aβ\(_{25-35}\) for 24 hours prior to [Ca\(^{2+}\)]\(_i\) being measured. Neurons were loaded with fura-2 by adding a 3 μM concentration of the acetoxymethyl ester form of fura-2 (fura-2 acetoxymethyl ester) in 0.3% dimethylsulfoxide (DMSO) to the media and incubating the cells at room temperature for 1 hour. Before beginning the experiments, the coverslips were rinsed with physiologic saline solution consisting of the following (in mM): 140 NaCl, 5.4 KCl, 25 HEPES, 20 glucose, 1.3 CaCl\(_2\) and 1.0 MgCl\(_2\) (pH adjusted to 7.4 with NaOH). For each cell, [Ca\(^{2+}\)]\(_i\) was measured once a second for a 20-second period, and the values were averaged to determine baseline [Ca\(^{2+}\)]\(_i\). The Aβ-induced increases in [Ca\(^{2+}\)]\(_i\) (Δ[Ca\(^{2+}\)]\(_i\)) in experiments using α-receptor antagonists were calculated by subtracting average [Ca\(^{2+}\)]\(_i\) measured in control cells for each condition tested (i.e., absence or presence of α-receptor ligand) from those recorded for the individual cells when Aβ was added under the same conditions. In one series of experiments, the acute effects of Aβ\(_{25-35}\) on neuronal [Ca\(^{2+}\)]\(_i\), were determined by first loading neurons with fura-2 and then administering Aβ\(_{25-35}\) via a rapid application system while measuring [Ca\(^{2+}\)]\(_i\).

### ROS and NO Imaging

Intracellular ROS and NO concentrations were measured in isolated cortical neurons using fluorometric digital microscopy (CoolSnap HQ2; Photometrics, Surrey, BC, Canada). Cells were plated as described above and incubated for 24 hours at 37°C in Neurobasal Complete containing 25 μM Aβ\(_{25-35}\) (±afobazole). Cultured cells were then incubated at 37°C for 30 minutes in 10 μM di-hydroethidiumbromide (DHE) with 0.25% DMSO to measure NO, both in Neurobasal Complete and with 25 μM Aβ\(_{25-35}\) (±afobazole). The fluorophores were then removed by rinsing coverslips with physiologic saline solution. DHE, and DAF-FM–loaded neurons were excited with light at 470 nm and 535 nm, respectively. Image analysis was performed using Nikon Elements software (Nikon, Melville, NY). No afobazole autofluorescence was observed at the wavelengths tested.

### Cytotoxicity Assay

Neurons plated on poly(L-lysine) coverslips were incubated at 37°C for 72 hours in Neurobasal Complete with 25 μM Aβ\(_{25-35}\), in the absence (Control) and presence of 100 μM afobazole or 100 μM L,3-di-iso-tolyguanidine (DTG). The coverslips were rinsed with phosphate-buffered saline (PBS) followed by 30-minute incubation at room temperature in PBS with 4 μl of 2 mM ethidium homodimer-1 (EthD-1) dissolved in 1:4 DMSO:H\(_2\)O solution. The coverslips were washed with PBS and deionized water, dried, and mounted on a microscope slide with Vectashield Hardset mounting medium (Vector Laboratories, Burlingame, CA). EthD-1-loaded cells were illuminated with light at 530 nm and visualized at 645 nm using a Zeiss Axioscope 2 equipped with a 20× objective. EthD-1-positive cells were identified and counted using Image-J in four random fields per slide, and the average for the fields was used as the value for the slide. For each experiment, a minimum of three slides were used, and the results of at least three experiments were averaged together for each condition tested.

### Immunocytochemistry

Neurons plated on poly(L-lysine) coverslips were incubated at 37°C for 24 hours in Neurobasal Complete with 25 μM Aβ\(_{25-35}\) in the absence and presence of 100 μM afobazole. The coverslips were rinsed with PBS followed by an ethanol step-wise fixation. The cells were permeabilized with 0.1% Triton X in PBS for 15 minutes and then rehydrated with PBS and washed with 0.5% bovine serum albumin (BSA) in PBS. Blocking was achieved with
45-minute incubation in 2% BSA, followed by multiple washes with 0.5% BSA. Primary antibodies were diluted in PBS with 0.5% BSA and applied to cells at 4°C for 24 hours. Primary antibody dilutions were as follows: Bax-1:20, caspase-3 1:25, and Bcl-2 1:100. The cells were then washed multiple times with 0.5% BSA in PBS and then incubated in secondary antibodies for 60 minutes at room temperature. Secondary antibodies were AlexaFluor 488 conjugated anti-mouse or anti-rabbit, as appropriate, both diluted at a ratio of 1:300 in PBS with 0.5% BSA. Following incubation in secondary antibodies, cells were washed with 0.5% BSA in PBS and then with PBS alone. Coverslips were rinsed with deionized water, inverted, and sealed onto a slide with VectaShield containing 4′,6-diamidino-2-phenylindole (DAPI). DAPI and the AlexaFluor 488–conjugated secondary antibodies were illuminated at 359 and 485 nm and visualized at 461 and 530 nm, respectively, using a Zeiss Axioskop 2 outfitted with a 40× objective. Images of DAPI and AlexaFluor 488–positive cells were counted and merged to demonstrate colocalization using ImageJ software. Four random fields per slide were used to determine an average value for the slide. For each experiment, a minimum of three slides were used, and the results of at least three experiments were averaged together.

**Compounds, Reagents, and Antibodies.** The following compounds and reagents were used in this investigation: Aβ25-35 (American Peptides, Sunnyvale, CA); DTG and N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine dihydrobromide (BD-1047) (Tocris Biosciences, Ellisville, MO); 9-[(3-cis-3,5-dimethyl-1-piperazinyl)propyl]-9H-carbazole dihydrochloride (rimcazole) (Sigma-Aldrich, St. Louis, MO); fura-2 acetoxymethyl ester, DHE, DAF-FM, and Live/Dead Viability/Cytotoxicity Kit (Life Technologies). The following antibodies were used: AlexaFluor 488 anti-mouse (A-11001) and anti-rabbit (A-11008) (Life Technologies); anti-Bax (ab57144), anti-activated caspase-3 (ab32351), and anti-Bcl-2 (ab2370) (Abcam, Cambridge, MA). Afobazole was generously provided by IBC Generium (Moscow, Russian Federation). Vehicles for drugs and reagents used were water (H2O), ethanol, or DMSO, and appropriate vehicle controls were carried out for each study.

**Data Analysis.** Data were analyzed using SigmaPlot 11 software (Systat Software, Inc., San Jose, CA). Data points represent peak means ± S.E.M. One- and two-way analyses of variance were used for determining significant differences for multiple group comparisons, as appropriate, followed by post hoc analysis using the Holm-Sidak test. Results were considered statistically significant if *P* < 0.05.

**Results**

Experiments were first carried out to determine the effects of Aβ25-35 on [Ca2+]i, in isolated cortical neurons from embryonic (E18) rats. Figure 1A shows representative traces of [Ca2+]i, measured in cortical neurons exposed to acute applications of 25 μM Aβ25-35 (<15 minutes). During this time period, the Aβ fragment did not significantly affect [Ca2+]i, in the neurons. To examine if extended incubation in Aβ25-35 alters [Ca2+]i, neurons were incubated from 1 to 24 hours in media containing 25 μM Aβ25-35. Figure 1B shows a bar graph of mean [Ca2+]i recorded from different groups of neurons incubated in Aβ25-35 for the indicated time points. The Aβ fragment produced a statistically significant increase in [Ca2+]i at all time points tested, with peak increases occurring 12 hours after Aβ25-35 application.

Given that activation of σ receptors can inhibit increases in intracellular Ca2+ under various pathologic conditions, we hypothesized that activation of σ receptors with the pan-selective σ-receptor agonists, afobazole or DTG, could affect the elevations in [Ca2+]i, triggered by incubation of cortical neurons in Aβ25-35. Cortical neurons were incubated in 25 μM Aβ25-35 for 24 hours in the absence and presence of 100 μM afobazole or 100 μM DTG (Fig. 2A). Following the 24-hour incubation with Aβ25-35, there was a statistically significant increase in [Ca2+]i, consistent with results shown in Fig. 1B. Incubation of neurons in media containing afobazole alone did not significantly affect neuronal [Ca2+]i, whereas incubation in afobazole blocked the Aβ25-35-evoked increases in [Ca2+]i by 58 ± 12% (Fig. 2A). In contrast, application of DTG (100 μM), failed to inhibit the Aβ25-35-evoked increase in [Ca2+]i, and instead, potentiated the response by 67 ± 21%.

The discrepancy between the results obtained for afobazole and DTG leave doubt as to the role of σ receptors in the regulation of Aβ25-35-induced increases in [Ca2+]i, by afobazole. Therefore, to test whether σ receptors are involved in these effects, selective inhibitors of σ-1 (BD-1047) and σ-2 (rimcazole) receptors were used (Matsumoto et al., 1995, Gilmore et al.,...
Fig. 2. Afobazole inhibits increases in \([\text{Ca}^{2+}]_i\), evoked by \(\text{A} \beta_{25-35}\). (A) Bar graph of mean ± S.E.M. \([\text{Ca}^{2+}]_i\), measured in neurons incubated for 24 hours in media in the absence (Control) or presence of 25 \(\mu\text{M} \text{A} \beta_{25-35}\) (\(\text{A} \beta\)) with or without 100 \(\mu\text{M}\) afobazole (Afob) or 100 \(\mu\text{M}\) DTG (DTG). Asterisks denote significant difference between Control and \(\text{A} \beta\) within the Media, Afob, and DTG groups \((P < 0.001\) for Media and DTG groups, \(P < 0.05\) for Afob group). Pound symbols denote significant difference from the Media group within the \(\text{A} \beta\) group \((P < 0.001\) for both). Dagger indicates significant difference between Afob and DTG groups within \(\text{A} \beta\) \((P < 0.01)\). For all groups, \(n = 125\) neurons. (B) Bar graph of mean ± S.E.M. \(\text{A} \beta\)-induced increase in \([\text{Ca}^{2+}]_i\), with change in \([\text{Ca}^{2+}]_i\), \(\Delta[\text{Ca}^{2+}]_i\) measured in neurons incubated for 12 hours in 25 \(\mu\text{M} \text{A} \beta_{25-35}\) alone (\(\text{A} \beta\)) or in 25 \(\mu\text{M} \text{A} \beta_{25-35}\) + 100 \(\mu\text{M}\) afobazole (Afob + Afob). \(\text{A} \beta\) or Afob + Afob were applied in media alone (Media), in media with 10 \(\mu\text{M}\) BD-1047 (BD), or in media with 300 nM rimcazole (Rim 0.3) or 10 \(\mu\text{M}\) rimcazole (Rim 10). Asterisks denote significant difference between \(\text{A} \beta\) and Afob + Afob within Media and rimcazole groups, respectively (for all \(P < 0.001\)). Pound symbols denote significant difference between BD-1047 (BD) and all other groups within \(\text{A} \beta\) and between BD-1047 and the Media and 300 nM rimcazole (Rim 0.3) within Afob + Afob, respectively \((P < 0.01\) for all). Dagger indicates significant difference between Rim 10 and Control groups within \(\text{A} \beta\) \((P < 0.001)\). For all groups in (B) and (C), \(n = 168\) neurons.

Cortical neurons were incubated in 25 \(\mu\text{M} \text{A} \beta_{25-35}\) for 12 hours in the absence and presence of 100 \(\mu\text{M}\) afobazole with and without BD-1047 or rimcazole (Fig. 2B). Application of afobazole reduced elevations in \([\text{Ca}^{2+}]_i\), predicted to interact with \(\sigma\)-1 receptors (Husbands et al., 1999), reduced \(\text{A} \beta_{25-35}\)-induced increases in \([\text{Ca}^{2+}]_i\) (Fig. 2B). However, \(\text{A} \beta_{25-35}\)-evoked increases in \([\text{Ca}^{2+}]_i\) observed in the cells incubated in 10 \(\mu\text{M}\) rimcazole + afobazole were not significantly different from those produced by the \(\text{A} \beta\) fragment in the presence of afobazole \((P = 0.179)\). To account for these direct effects of the antagonists on \(\text{A} \beta_{25-35}\)-evoked elevations in \([\text{Ca}^{2+}]_i\), we normalized the increases in \([\text{Ca}^{2+}]_i\) observed when afobazole was added along with the \(\sigma\) receptor antagonist to those observed when \(\text{A} \beta\) was added with the antagonist alone. The mean normalized responses observed for the different conditions are shown in Fig. 2C. The effects of afobazole were blocked by both BD 1047 and the higher concentration of rimcazole (10 \(\mu\text{M}\), rimcazole [Rim 10]), but not by the lower concentrations of rimcazole (300 nM, rimcazole [Rim 0.3]) (Fig. 2C). Therefore, inhibition of \(\sigma\)-1, but not \(\sigma\)-2, receptors lessened the effects of afobazole.

Cortical neuron \([\text{Ca}^{2+}]_i\) overload has been shown to enhance production of reactive oxygen species and nitric oxide, with the latter molecule being converted to reactive nitrogen species in the presence of ROS. Activation of \(\sigma\) receptors has been
associated with changes in both ROS and nitric oxide levels. Thus, we evaluated if afobazole preservation of \([\text{Ca}^{2+}]\), homeostasis consequently affects ROS and/or nitric oxide levels following exposure to \(\text{A}_25-35\). Figure 3A shows a bar graph of the average DHE fluorescence intensities measured in neurons in response to 24-hour incubation in \(\text{A}_25-35\) in the absence and presence of 100 \(\mu\text{M}\) afobazole. Greater DHE fluorescence was observed in neurons exposed to \(\text{A}_25-35\) relative to media alone, indicating an \(\alpha\beta\)-induced increase in ROS production (Fig. 3A). Incubation of the neurons in afobazole alone also produced a small but significant increase in neuronal ROS, and a further increase in ROS was noted when \(\text{A}_25\) was coapplied with afobazole (Fig. 3A). NO levels were also measured in neurons exposed to \(\text{A}_25-35\) for 24 hours. Incubation of neurons in \(\text{A}_25-35\) significantly enhanced neuronal NO levels. In contrast to the results obtained for ROS, afobazole alone had no effects on basal neuronal NO content. However, the \(\alpha\) agonist significantly decreased \(\text{A}_25-35\)-evoked elevations in NO by 53 ± 5% (Fig. 3B).

Afobazole suppression of \(\text{A}_25-35\)-evoked \([\text{Ca}^{2+}]\) disorganization and NO production suggests that this compound may be neuroprotective in this injury model. Thus, experiments were conducted to ascertain if afobazole can reduce \(\text{A}_25-35\) cytotoxicity and enhance cell survival following exposure of cortical neurons to this \(\alpha\beta\) fragment. For comparison, a second \(\alpha\) receptor agonist, DTG, which has been shown to augment survival in other neuronal injury models but does not block \(\text{A}_25-35\)-elicited \([\text{Ca}^{2+}]\) increases, was included. Neurons were incubated for 72 hours in 25 \(\mu\text{M}\) \(\text{A}_25-35\) in the absence and presence of 100 \(\mu\text{M}\) afobazole or 100 \(\mu\text{M}\) DTG, and cell survival determined using EthD-1 to identify apoptotic cells. Fig. 4A shows representative photomicrographs of neurons labeled with EthD-1. While EthD-1 staining was noted in all groups, a higher number of cells were observed when \(\text{A}_25-35\) was applied alone or in the presence of DTG (Fig. 4A, ii and vi), but not when coapplied with afobazole (Fig. 4Aiv). In identical experiments, incubation in \(\text{A}_25-35\) resulted in a 130 ± 6% increase in the percentage of cells exhibiting positive labeling with EthD-1, relative to control (media alone, Fig. 4B). When afobazole (100 \(\mu\text{M}\)) was applied alone, there was no change in the percentage of dead cells noted. However, application of afobazole alone with the \(\text{A}_25-35\) resulted in an 88 ± 4% reduction in the percentage of EthD-1 positive cells relative to the \(\alpha\beta\) fragment alone group, which was statistically significant (Fig. 4B). In contrast, DTG alone increased the percentage of EthD-1 positive cells compared with control (Fig. 4B). Moreover, DTG failed to reduce the percentage of cells exhibiting EthD-1 staining when coincubated with \(\text{A}_25-35\) such that the number of apoptotic cells seen with \(\text{A}_25-35\) alone and \(\text{A}_25-35\) + DTG were similar (Fig. 4B).

Experiments were next focused on identifying underlying molecular mechanisms contributing to the enhanced survival of cortical neurons following afobazole application. Previous studies have shown that \(\alpha\)-1-receptor activation by the selective agonist [2S-(2\(\alpha\),6\(\alpha\),11\(R\)]\(-1\)-2,3,4,5,6-hexahydro-6,11-dimethyl-3-(2-propenyl)-2,6-methano-3-benzazocin-8-ol hydrochloride can protect RGC-5 cells from glutamate-induced apoptosis by reducing expression of the proapoptotic gene product Bax (Tchedre and Yorio, 2008). To determine whether a similar pathway is involved in afobazole-mediated neuroprotection during \(\text{A}_25-35\) exposure, the cellular expression of this protein was determined. Representative photomicrographs of cells colabeled with DAPI and an anti-Bax antibody are shown in Fig. 5A. Although Bax is detected in control neurons (Fig. 5Ai), there is a marked increase following incubation in \(\text{A}_25-35\) (Fig. 5Aii). Bax is also expressed in low levels in neurons exposed to afobazole alone (Fig. 5Aiii), and there is only a modest increase when neurons are incubated in both 100 \(\mu\text{M}\) afobazole and the \(\alpha\beta\) fragment (Fig. 5Aiv). Analysis of images collected from multiple experiments shows that Bax is detected in less than half of the control cells, but following exposure to \(\text{A}_25-35\), there is a 100 ± 5% increase in the number of cells testing positive for this proapoptotic gene (Fig. 5B). Application of afobazole reduces Bax expression below that observed in control cells by 13 ± 5%, and the increase in expression occurring after \(\text{A}_25-35\) incubation is reduced to 58 ± 5% (Fig. 5B). The net result is a 39 ± 5% reduction in the total number of cells expressing Bax in response to \(\text{A}_25-35\) when afobazole was applied.

Given that \(\alpha\) receptors have also been linked to decreased caspase-3 expression (Tchedre and Yorio, 2008), immunocytochemistry was also used to examine how afobazole affects the levels of the active form of this death protease after neuronal exposure to \(\text{A}_25-35\). Representative photomicrographs of cells

![Fig. 3. Afobazole does not inhibit \(\text{A}_25-35\) evoked ROS production but does inhibit nitric oxide production in neurons.](https://example.com/fig3.png)
labeled with DAPI and anti-activated caspase-3 antibody show an increase in the number of caspase-3–positive neurons 24 hours after incubation in Aβ25–35 relative to control (Fig. 6A, i and ii). Neuronal expression of the active form of caspase-3 was not affected by incubation of the cells in 100 μM afobazole alone, but coapplication of afobazole with Aβ25–35 significantly lowered activated caspase-3 expression when compared with cells treated only with Aβ25–35 (Fig. 6A, iii and iv). Figure 6B shows Afobazole, but not DTG, reduces neuronal death produced by application of Aβ25–35. (A) Photomicrographs showing EthD-1 labeling (red), a marker of cell death, observed following 72-hour incubation of neurons in media alone (i), or media containing 25 μM Aβ25–35 (ii), 100 μM afobazole (iii), 25 μM Aβ + 100 μM afobazole (iv), 100 μM DTG (v), or 25 μM Aβ + 100 μM DTG (vi). Fields of view shown are representative regions within larger images used to calculate numbers of neurons labeled with EthD-1. (B) Bar graph of relative neuronal death observed for the same conditions as in (A). Data were normalized to the average number of EthD1-positive cells observed with media alone (i.e., no Aβ or σ ligand). Asterisks denote significant difference between Control and Aβ within Media and DTG groups, respectively (P < 0.001 for both). Pound symbol indicates significant difference between Afob and the other groups within Aβ (P < 0.001), and dagger indicates significant difference between DTG and the other groups within Control (P < 0.05). For all groups, n ≥ 12. Scale bar in (i) is 20 μm.

Fig. 5. Afobazole prevents upregulation of the proapoptotic gene product, Bax, caused by application of Aβ25–35 in neurons. (A) Photomicrographs of merged images of cultured neurons exposed for 24 hours to media alone (Control) (i), or media containing 25 μM Aβ25–35 (Aβ) (ii), 100 μM afobazole (Afob) (iii), or 25 μM Aβ25–35 + 100 μM afobazole (Aβ + Afob) (iv). Neurons were double-labeled with DAPI (blue) and anti-Bax antibody (green). Fields of view shown are representative regions within larger images used to calculate Bax expression. (B) Bar graph of mean percent of Bax-positive neurons observed in multiple experiments in which cells were exposed to the same conditions described in (A). Data are expressed as mean percentage ± S.E.M. of the total number of neurons staining positive for DAPI. Asterisks denote significant difference between Control and Aβ within Media and Afob groups, respectively (P < 0.001). Pound symbols indicate significant difference between Afob and Media groups within Control (P < 0.05) and Aβ (P < 0.001). Scale bar in (i) is 10 μm, and n = 9 for all groups in (B).
a bar graph of mean ± S.E.M. percentages of active caspase-3-positive neurons detected from multiple experiments using the same conditions as in Fig. 6A. There is a statistically significant upregulation of activated caspase-3 expression following Aβ25-35 application, which results in a 104 ± 7% increase in the percentage of neurons testing positive for the death protease. However, treatment with afobazole reduces the increased cellular expression of activated caspase-3 produced by Aβ25-35 by 89 ± 7% (Fig. 6B). Neuronal expression of active caspase-3 was not statistically different (P = 0.20).

Activation of σ receptors has been shown to provide neuroprotection against toxicity produced by the human immunodeficiency virus–1 protein gp120 via upregulation of the antiapoptotic gene Bcl-2 (Zhang et al., 2012). Therefore, we examined the possible involvement of Bcl-2 in the protection provided by afobazole against Aβ25-35-induced cytotoxicity. A significant number of neurons were found to express Bcl-2 in our culture model under all conditions tested at both 24 and 48 hours (Fig. 7A, i–vii). Quantification of the results indicates that 69 ± 6% of control neurons expressed Bcl-2, and that incubation in 25 μM Aβ25-35 for 24 hours increased this number to 90 ± 2% (Fig. 7B). The addition of afobazole alone also produced a 24 ± 2% increase in the percentage of Bcl-2 expressing cells, which was statistically significant when compared with the control (Fig. 7B). However, after a 24-hour incubation, no further increase in Bcl-2 was noted when afobazole was applied with Aβ25-35 compared with incubation in Aβ25-35 alone. In contrast, increasing the incubation time of the cells in Aβ25-35 to 48 hours resulted in reduction in the number of cells showing positive labeling for Bcl-2 expression, with Bcl-2 being observed in 71 ± 1% of cells incubated in media alone and 46 ± 2% of the cells incubated in media containing the Aβ fragment (Fig. 7B). Extending the application time of afobazole to 48 hours continued to produce a similar increase in Bcl-2 expression as seen with the 24-hour incubation, with 85 ± 1% of the afobazole-treated neurons showing Bcl-2 expression. However, the addition of afobazole to Aβ25-35 prevented the Aβ-induced suppression of Bcl-2 seen at 48 hours, with Bcl-2 being observed in 81 ± 1% of the neurons exposed to Aβ25-35 in the presence of this drug (Fig. 7B). In fact, in cultures exposed to afobazole + Aβ there was a 14 ± 2% increase in the number of neurons testing positive for Bcl-2 expression compared with control (no Aβ or afobazole).

**Discussion**

The major finding reported in this study is that afobazole, acting via σ-1 receptors, provides neuroprotection against Aβ25-35-induced toxicity in cortical neurons. Activation of σ-1 receptors by afobazole appears to impinge on multiple molecular mechanisms associated with the demise of neurons. First, afobazole, acting via σ-1 receptors, decreases neuronal [Ca2+]i dyshomeostasis resulting from prolonged exposure (1–24 hours) of the cells to Aβ25-35. Second, afobazole mitigates Aβ25-35-evoked elevations in nitric oxide production in cortical neurons that coincides with increases in ROS production, thus nitrosative stress is likely lessened. Finally, afobazole blunts the proapoptotic signaling induced by Aβ25-35 by inhibiting the upregulation of the proapoptotic gene product Bax and the death protease, caspase-3, while preventing long-term downregulation of the antiapoptotic protein, Bcl-2.

**Fig. 6.** Afobazole reduces expression of activated caspase-3 in response to application of Aβ25-35 in neurons. (A) Photomicrographs of merged images of cultured neurons exposed for 24 hours to media alone (Control) (i), or media containing 25 μM Aβ25-35 (Aβ) (ii), 100 μM afobazole (Afob) (iii), or 25 μM Aβ25-35 + 100 μM afobazole (Aβ + Afob) (iv). Neurons were double-labeled with DAPI (blue) and anti-active caspase-3 antibody (green). Fields of view of each photomicrograph are representative regions within larger images used to calculate caspase-3 expression. (B) Bar graph of mean percentage ± S.E.M. of neurons testing positive for the active form of caspase-3 in experiments in which the cells were exposed to the same conditions as (A). Asterisk denotes significant difference between Control and Aβ within Media (P < 0.001). Pound symbol indicates significant difference between Media and Afob within Aβ (P < 0.001). Scale bar in (i) is 10 μm, and n = 9 for all groups in (B).
In our culture system, Aβ25–35 was found to produce increases in [Ca$^{2+}$]$_i$ after cells were incubated in the Aβ fragment for $\approx$1 hour, with short applications $\leq$15 minutes failing to alter [Ca$^{2+}$]$_i$. Maximal increases in [Ca$^{2+}$]$_i$ were observed after 12-hour incubation of the neurons in Aβ25–35, and [Ca$^{2+}$]$_i$ remained significantly elevated even after 24-hour incubation in the Aβ fragment. The observed 2-fold increase in [Ca$^{2+}$]$_i$ caused by Aβ25–35 is similar to that previously reported for rat cortical neurons incubated for 24 hours in Aβ25–35 (Ferreiro et al., 2004). Incubation of neurons in 100 μM afobazole reduced Aβ25–35-evoked increases in [Ca$^{2+}$]$_i$, by $\sim$60%. This decrease in [Ca$^{2+}$]$_i$ is similar to that observed when afobazole was used to inhibit [Ca$^{2+}$]$_i$ increases produced in cortical neurons by acidosis (IC$_{50}$ = 164 μM) (Cuevas et al., 2011a). Reducing Aβ25–35 perturbation of [Ca$^{2+}$]$_i$ is likely to promote neuronal survival, since [Ca$^{2+}$]$_i$ destabilization has been linked to cell death in AD (Mattson and Chan, 2003). Moreover, the Aβ25–35-induced changes in [Ca$^{2+}$]$_i$ precede proapoptotic signals in the model used here (e.g., decrease in Bcl-2 levels occurring 48 hours after Aβ25–35 application).

The inhibition of Aβ25–35-elicted increases in [Ca$^{2+}$]$_i$ by afobazole is due to activation of $\sigma$-1, but not $\sigma$-2, receptors. Incubation in the $\sigma$-1-receptor antagonist BD-1047 (Matsumoto et al., 2004) reduced the effects of afobazole by $\sim$40%. The concentration and degree of block reported here is consistent with previous studies showing BD-1047 inhibition of $\sigma$-1-receptor-mediated effects in cortical neurons and retinal ganglion cells (Katnik et al., 2006, Cuevas et al., 2011a, Mueller et al., 2013). The lack of involvement of $\sigma$-2 receptors is confirmed by results obtained using the $\sigma$-2 receptor antagonist rimcazole (Gilmore et al., 2004). Rimcazole binds to $\sigma$-2 receptors with high nanomolar affinity, but to the $\sigma$-1 receptor with low micromolar affinity (Ferris et al., 1986, Husbands et al., 1999, Rybczynska et al., 2008). Given that 300 nM rimcazole failed to inhibit the actions of afobazole on Aβ25–35-evoked increases in [Ca$^{2+}$]$_i$, but that the higher concentration of the drug reduced these effects, it is unlikely that afobazole activates $\sigma$-2 receptors to suppress Aβ25–35-evoked increases in [Ca$^{2+}$]$_i$. Surprisingly, a second pan-selective $\sigma$ receptor agonist, DTG, failed to mimic the effects of afobazole on Aβ25–35-evoked [Ca$^{2+}$]$_i$ perturbation. DTG and afobazole both block acidosis- and ischemia-induced Ca$^{2+}$ overload in neurons to a similar extent (Cuevas et al., 2011a). It was shown that ischemia-induced [Ca$^{2+}$]$_i$ decreases were due primarily to Ca$^{2+}$ influxes through voltage-gated Ca$^{2+}$ channels and NMDA receptors which were regulated by $\sigma$ receptor activation (Katnik et al., 2006). However, the difference between afobazole and DTG effects on Aβ modulation of [Ca$^{2+}$]$_i$ suggest these long-term [Ca$^{2+}$]$_i$ increases involve distinct mechanisms from those producing ischemia-induced [Ca$^{2+}$]$_i$ overload. Additionally, the lack of a DTG effect on Aβ-evoked [Ca$^{2+}$]$_i$ overload suggests that not all $\sigma$-1 agonists may promote...
neuronal survival in this model. This hypothesis is further supported by our observation that DTG fails to decrease neuronal apoptosis following 24-hour incubation with Aβ25–35. The difference in responses between afobazole and DTG may be due to off-target effects of DTG counteracting the benefits of this pan-selective sigma agonist. Such a possibility needs to be explored further.

It has been suggested that one of the downstream consequences of [Ca2+]i dysregulation in neurons caused by Aβ25–35 is the production of ROS (Ekinci et al., 2000, Abramov et al., 2004). Afobazole has been shown to reduce ROS accumulation in rat brains after focal ischemia (Silkina et al., 2004). However, data presented here show that afobazole does not block the ROS increases caused by the Aβ fragment. It has been suggested that Aβ25–35-induced cell death by acting downstream of the observed ROS increase.

Aβ25–35 has been shown to increase nitric oxide synthase (NOS) activity and produce neuronal injury via nitrosative stress (Parks et al., 2001, Cho et al., 2009). Aβ25–35-evoked increases in NO were significantly decreased following afobazole application. Inhibition of inducible NOS has been proposed as a mechanism by which activation of σ-1 receptors is neuroprotective after stroke (Vagnerova et al., 2006). Similarly, functional downregulation of neuronal NOS has been shown to contribute to σ receptor neuroprotection following ischemia in striatal neurons (Yang et al., 2010). Because Aβ25–35 appears to increase both inducible NOS and neuronal NOS in neurons (Limon et al., 2009), activation of σ receptors by afobazole is predicted to reduce NO production by both of these enzymes and will ultimately contribute to protection from Aβ25–35-induced injury.

One of the proapoptotic proteins upregulated in neurons both by the pathologic sequelae induced by Aβ25–35 application and in patients with Alzheimer’s disease is Bax (Paradis et al., 1996, Yao et al., 2005). Consistent with previous findings, the current study shows that the number of Bax-positive neurons is significantly increased following 24-hour incubation in Aβ25–35. However, when afobazole is applied in combination with Aβ25–35, Bax upregulation is diminished significantly. It has also been shown that the σ-1 agonist, PRE-084, can reduce apoptosis in cortical neurons caused by Aβ25–35 via reduction in Bax (Marrazzo et al., 2005). However, glutamatergic neurotransmission was blocked in those experiments (Marrazzo et al., 2005). Glutamatergic transmission is a factor in Aβ25–35-induced cell death, and elevated glutamate levels have been shown to induce Bax upregulation in neurons (Schelman et al., 2004, Revett et al., 2013). Thus, our data suggest that σ receptor activation by afobazole can reduce Bax expression and concomitant cell death in the presence of Aβ25–35 even when glutamatergic transmission is not blocked.

In addition to decreasing Bax levels, afobazole effectively reduced activated caspase-3 expression in response to Aβ25–35. The active form of caspase-3 has been implicated in the loss of hippocampal neurons in Alzheimer’s disease (Selznick et al., 1999). In addition to serving as a late event involved in neuronal death during Alzheimer’s disease, caspase activation may be an early event that promotes the pathology of AD. Several caspases, including caspase-3, have been shown to cause pathologic τ filament assembly in neurons, which underlies AD etiology (Gamblin et al., 2003). Selective inhibition of caspase-3 has been shown to protect neurons from Aβ25–35-induced apoptosis (Allen et al., 2001). Activation of σ receptors has been shown to decrease caspase-3 and protect cortical neurons and RGC-5 cells against excitotoxicity (Tchedre and Yorio, 2008, Griesmaier et al., 2012). Such a mechanism is likely to contribute to afobazole neuroprotection from Aβ25–35 toxicity and may in part explain the reduced neuronal death observed here.

The neuroprotective properties of afobazole following exposure to Aβ25–35 also involve long-term upregulation of Bcl-2. Our data indicate that there is a biphasic change in Bcl-2 levels in neurons following application of Aβ25–35 with an initial increase at 24 hours followed by a suppression of Bcl-2 expression at 48 hours. A similar transient expression pattern was previously reported in rat hippocampal neurons exposed to Aβ25–35 (Kim et al., 1998). It was proposed that this initial increase in Bcl-2 expression is a nonsustainable cellular response to the apoptotic pathways induced by Aβ (Kim et al., 1998). Our observations indicate that when afobazole is applied along with Aβ25–35, Bcl-2 levels do not drop below control at the 48-hour time point. Previous studies have shown that Bcl-2 is a target for σ-receptor-mediated neuroprotection. The human immunodeficiency virus–1 protein gp120 was shown to downregulate Bcl-2 in cortical neurons, but activation of σ receptors with the σ ligand, 4-phenyl-1-(4-phenylbutyl) piperidine, increased Bcl-2 expression (Zhang et al., 2012). 4-Phenyl-1-(4-phenylbutyl) piperidine was shown to have a similar effect in a glutamate excitotoxicity model (Yang et al., 2007), suggesting that Bcl-2 upregulation may occur after σ-receptor stimulation under various pathophysiological conditions.

In conclusion, our experiments show that afobazole can decrease Aβ25–35-evoked neuronal apoptosis by activating σ-1 receptors. The activation of σ-1 receptors prevents Aβ25–35 induced [Ca2+]i overload and is associated with a decrease in NO levels. The molecular mechanisms mediating neuroprotection by afobazole include downregulation of both Bax and activated caspase-3, and long-term upregulation of Bcl-2. Given the importance of direct Aβ neurotoxicity in the etiology of Alzheimer’s disease, the findings reported here suggest that σ-1 receptors are a putative target for AD therapy and that the σ receptor agonist, afobazole, in particular, shows significant potential.

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Authorship Contributions:

Participated in research design: Behensky, Yasny, Shuster, Seredenin, Petrov, Cuevas.

Conducted experiments: Behensky.

Performed data analysis: Behensky.

Wrote or contributed to the writing of the manuscript: Behensky, Yasny, Cuevas.