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A prototypical Sigma-1 receptor antagonist protects against brain ischemia

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Abstract

Previous studies indicate that the Sigma-1 ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) protects the brain from ischemia. Less clear is whether protection is mediated by agonism or antagonism of the Sigma-1 receptor, and whether drugs already in use for other indications and that interact with the Sigma-1 receptor might also prevent oxidative damage due to conditions such as cerebral ischemic stroke. The antipsychotic drug haloperidol is an antagonist of Sigma-1 receptors and in this study it potently protects against oxidative stress-related cell death *in vitro* at low concentrations. The protective potency of haloperidol and a number of other butyrophenone compounds positively correlate with their affinity for a cloned Sigma-1 receptor, and the protection is mimicked by a Sigma-1 receptor-selective antagonist (BD1063), but not an agonist (PRE-084). *In vivo*, an acute low dose (0.05 mg/kg s.c.) of haloperidol reduces by half the ischemic lesion volume induced by a transient middle cerebral artery occlusion. These *in vitro* and *in vivo* pre-clinical results suggest that a low dose of acutely administered haloperidol might have a novel application as a protective agent against ischemic cerebral stroke and other types of brain injury with an ischemic component.

Keywords

Sigma receptor; Neuroprotection; Oxidative stress; Acute stroke treatment; Cerebral ischemic stroke; Antipsychotic; Neuroleptic

1. Introduction

High rates of disabling ischemic stroke in a rapidly increasing elderly population have made cerebral ischemic stroke one of the nation's most urgent health care concerns. Over 80% of strokes are classified as ischemic strokes and approximately 94% of those presenting are 45 years of age or older (Grau et al., 2001). Among the number of modifiable and non-modifiable risk factors for ischemic stroke are various surgical procedures, which pose a considerable risk (2% to 11%) (Wong et al., 2000; Baskett et al., 2005). However, the risks

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due to surgery are also the most predictable and thus may be most amenable to acute intervention strategies.

Some promising preclinical pharmacotherapies for limiting the damage induced by oxidative stress related to ischemic cerebral stroke are antioxidants (Liu et al., 2002; Bhavnani, 2003; Calabrese et al., 2003; Granot and Kohen, 2004; also see Margaill et al., 2005), NMDA receptor antagonists (Farber et al., 2002; Petty et al., 2003; Li et al., 2004), and the Sigma-1 receptor ligand 4-phenyl-1-(4-phenylbutyl) piperidine (PPBP) (Takahashi et al., 1995, 1996; Goyagi et al., 2001, 2003). Drugs targeting Sigma-1 receptors appear to be a relatively attractive therapeutic protection strategy, in comparison to NMDA receptor antagonists that have a narrow therapeutic window or anti-oxidants that work only at high doses. However, it is not clear whether the Sigma-1 receptor ligand PPBP protects against ischemic stroke by activating or inhibiting Sigma-1 receptor function.

Our initial studies were designed to further explore the protective role of Sigma-1 receptors by correlating the binding affinities of compounds for the cloned Sigma-1 receptor with their protective potencies against oxidative stress-induced cell death *in vitro*. Furthermore, compounds that are known to be selective agonists or antagonists of Sigma-1 receptors were utilized as probes to determine whether the protection against oxidative stress is due to activating or inhibiting Sigma-1 receptors. Since the *in vitro* results indicated that inhibiting, but not activating, the Sigma-1 receptor prevents oxidative stress-induced cell death, our subsequent studies were designed to investigate whether an established Sigma-1 receptor antagonist with great translational potential, haloperidol, could protect against ischemic cerebral stroke in an animal model. The results indicate that an acute low dose of haloperidol (0.05 mg/kg s.c.) reduces ischemic lesion volume in rats by 50%. Our *in vitro* and *in vivo* studies suggest that Sigma-1 receptor antagonists, but not agonists, protect against oxidative stress-induced cell death and that the Sigma-1 receptor antagonist haloperidol might be repurposed for the acute treatment of ischemic cerebral stroke.

2. Results

The potential of Sigma-1 receptors as therapeutic targets for protecting neurons against oxidative stress was assessed by screening agonists and antagonist *in vitro*. Application of extracellular glutamate to the immortalized mouse hippocampal cell line HT-22 was selected as our *in vitro* model, because others have demonstrated that this produces a state of oxidative stress (Ishige et al., 2001; Choi et al., 2003; Tomizawa et al., 2005). Even though we did not demonstrate it in the current study, high extracellular glutamate engenders oxidative stress in HT-22 cells by reversing the glutamate/cystine-antiporter, which depletes intracellular cystine needed for the production of the endogenous antioxidant glutathione leading to an increase in reactive oxygen species (Li et al., 1998; Ishige et al., 2001; Tomizawa et al., 2005). In this *in vitro* model of oxidative stress, the Sigma-1 receptor-selective antagonist BD1063, but not the selective agonist PRE-084, was protective (Figs. 1A and C). Like BD1063, the prototypical Sigma-1 receptor antagonist and butyrophenone antipsychotic drug haloperidol also potently protected HT-22 cells (Fig. 1B). Membranes prepared from HT-22 cells specifically bound the Sigma-1 receptor preferring radioligand

[^{3}H]-(+)-pentazocine with high affinity in a dose-dependent and saturable manner (Fig. 2, K_D =4.2±1.7 nM and B_{max} =5.0±0.83 pmol/mg membrane protein).

To correlate the protective potency of compounds with their affinity for the Sigma-1 receptor, an unambiguous assay was developed (Lee et al., in press) that measures the affinity of ligands for a cloned Sigma-1 receptor expressed in a null background (Vilner et al., 1995; Seth et al., 1998; Yamamoto et al., 1999; Shamsul Ola et al., 2002). Stable expression of the full-length, cloned Sigma-1 receptor in MCF-7 cells resulted in very high levels of high affinity [^{3}H]-(+)-pentazocine binding (i.e., K_D = 3.7±0.50 nM and B_{max} =108±13.7 pmol/mg membrane protein). Using this assay system, a positive correlation was found between the *in vitro* protective potency of nine anti-psychotic drugs belonging to the butyrophenone structural class and their affinities for the Sigma-1 receptor (Fig. 3A and Table 1). Clear substructural requirements for Sigma-1 receptor-mediated protection by butyrophenones were also evident: potent protection and high affinity binding to the Sigma-1 receptor required the presence of both a 4-linked phenyl and an electronegative moiety at position one along the butyl chain (Fig. 3B). For example, haloperidol, reduced haloperidol, and trifluperidol had low nanomolar affinities for the Sigma-1 receptor and low nanomolar neuroprotective potencies and both of these compounds possess a 4-linked phenyl and an electronegative group at position 1 along the butyl chain. Droperidol and spiperone had micromolar affinities for the Sigma-1 receptor and micromolar neuroprotective potencies and both of these compounds lack a 4-linked phenyl. Penfluridol lacks an electronegative group at position 1 along the butyl chain and it had a mid range nanomolar affinity for the Sigma-1 receptor and a mid range nanomolar neuroprotective potency.

Since the *in vitro* screening suggested that protection against oxidative stress-related cell death is mediated via Sigma-1 receptor antagonism, we investigated whether the antipsychotic drug haloperidol could produce a similar protection *in vivo*. Haloperidol was selected for the following reasons. It is a Sigma-1 receptor antagonist, it is a butyrophenone possessing the critical substructural features required for *in vitro* neuroprotection, and it would have considerable translational potential for acute applications due to its long history of use in humans. A rat tMCAO model of ischemic cerebral stroke was selected to investigate the protective effect of a low dose of haloperidol, because it is a well-established *in vivo* model of oxidative stress (Dirnagl et al., 1999; Warner et al., 2004; Zhan and Yang, 2006). Ovariectomized female rats were utilized to eliminate the protective effects of endogenous cycling estrogens, which reduce infarct size. An acute low dose of haloperidol (0.05 mg/kg) administered subcutaneously immediately following the induction of a tMCAO provided a 50% reduction in infarct volume assessed 24 h after reperfusion (Fig. 4).

3. Discussion

In previous studies, Sigma-1 ligands have been shown to protect against chemical ischemia-induced and glutamate-induced toxicity in rat cortical (DeCoster et al., 1995; Nishikawa et al., 2000; Kume et al., 2002) and ganglion cell cultures (Martin et al., 2004). However, both Sigma-1-selective agonists like (+)-SKF 10,047 and Sigma-1 antagonists like haloperidol were shown to be protective and both have potencies around 1–4 μM. The necessity for such

extremely high concentrations (micromolar) and the finding that both an agonist and an antagonist produced the same effect is consistent with a direct NMDA receptor blocking action for both (+)-SKF10,047 and haloperidol (Maurice and Lockhart, 1997; Nishikawa et al., 2000; Kume et al., 2002). Thus, while it is true that haloperidol can antagonize both Sigma-1 receptors and NMDA receptors, haloperidol's affinity for NMDA receptors (>1 μM) is three orders of magnitude lower than for Sigma-1 receptors (Fletcher et al., 1995; Coughenour and Cordon, 1977; Whittemore et al., 1997; Gallagher et al., 1998; Shim et al., 1999; Hayashi et al., 1999; Bowen et al., 1990; Ganapathy et al., 1999; Nishikawa et al., 2000). Consequently, the concentration of haloperidol used in each study is a critical factor when considering its potential receptor targets. This does not mean that Sigma-1 receptors cannot indirectly mediate excitotoxicity evoked by NMDA receptor stimulation, as this has been demonstrated (Bhardwaj et al., 1998), rather only that the protective effect of low nanomolar concentrations of haloperidol cannot be due to a direct blockade of NMDA receptors.

Our *in vitro* results for haloperidol are consistent with the Sigma-1 receptor being the protective molecular target and antagonism of this target being the pharmacological mechanism of action. Saturation isotherm studies indicated an abundance of an endogenous receptor in the HT-22 cell line, whose high affinity for [^{3}H]-(+)-pentazocine is within the range expected for Sigma-1 receptors (Seth et al., 1998; Mei and Pasternak, 2001) and the same as that for the cloned Sigma-1 receptor expressed in MCF-7 cells reported here. In our studies, haloperidol strongly protected HT-22 cells against glutamate-induced oxidative stress at a concentration of approximately 1 nM. Consequently, it is unlikely that the protective effect we observed could be mediated by NMDA receptors, even though high concentrations of haloperidol (and trifluperidol) have been reported to block NMDA receptors and NMDA receptors can provide neuroprotection (Li et al., 2004). Additionally, it has been reported that the HT-22 cells utilized for our cellular model of oxidative stress are devoid of NMDA receptors (Zaulyanov et al., 1999; Ishige et al., 2001). D2 dopamine or serotonin 5HT2A receptors, both of which have high affinity for haloperidol, are also not the likely protective targets for haloperidol, because other antagonists of these receptor systems do not mimic the protection afforded by haloperidol (data not shown). Furthermore, reduced haloperidol (metabolite II) retains high affinity for the Sigma-1 receptor and strong protective potency, even though its affinity for the D2 dopamine receptor is drastically reduced (~350-fold data not shown). Moreover, other butyrophenones have a protective potency that is positively correlated with their affinity for the cloned Sigma-1 receptor. Finally, the Sigma-1 receptor-selective antagonist BD1063 mimics the strong protection observed for haloperidol, but the Sigma-1 receptor-selective agonist PRE-084 does not. All of these lines of evidence suggest that the *in vitro* protective effect of haloperidol is mediated via the Sigma-1 receptor.

The *in vivo* protective potential of acutely administered haloperidol was tested in an established tMCAO model that utilizes young ovariectomized female rats and ischemic lesion volume as the neuroprotective measure. This *in vivo* model was selected for the following reasons. The stroke procedure, including monofilament size, occlusion times, and expected survival rates and degree of ischemic damage have been extensively characterized in young ovariectomized female rats (Simpkins et al., 1997; Shi et al., 1997; Zhang et al.,

1998). Specifically, removing the ovaries results in large and reproducible ischemic lesion volumes at shorter occlusion times (i.e., 1 h vs. 2 h). This is due to the loss of the natural protective effect of endogenous cycling estrogens that are neuroprotective when replaced (Simpkins et al., 1997; Shi et al., 1997; Zhang et al., 1998). Preliminary studies in aged (post-menopausal) females resulted in a high degree of death and highly variable lesion volumes (Markgraf et al., 1994; Markgraf et al., 1997; Yang and Simpkins, in press). Reduction in ischemic lesion volume after 24 h was used as the primary measure of protection, because previous studies have shown it to be a more accurate measure than functional outcomes, which are more variable and change with time (Simpkins et al., 1997; Markgraf et al., 1994, 1997; Yang and Simpkins, in press). In this initial exploratory study, haloperidol was administered acutely and as a single dose, in order to demonstrate proof of concept that might relate to a potential translational application, i.e., acute phase treatments. The low dose relates to *in vivo* discrimination between Sigma-1 receptors and NMDA receptors.

The *in vivo* protection observed for haloperidol is not likely to be due to excitotoxic receptor blockade, because the protective dose is orders of magnitude lower than what would be needed to block NMDA receptors. The acute low dose of haloperidol needed to provide a substantial protection against transient ischemic cerebral stroke (i.e., 0.05 mg/kg s.c.) is the same dose that results in an *in vivo* D2 dopamine receptor occupancy of about 65–70% (Kapur et al., 2000; Wadenberg et al., 2000). This low dose produces behaviors in rats indicative of antipsychotic action in humans, but not those indicative of extrapyramidal side effects, even when administered chronically. Our rationale for this test dose relates to the possible repurposing of butyrophenone antipsychotics with the appropriate substructural features as protectants to treat acute ischemic cerebral stroke with limited risk of extrapyramidal side effects. Furthermore, since haloperidol has a similar affinity for both D2 dopamine receptors and Sigma-1 receptors, it was possible to select a dose that by analogy would result in significant occupancy of Sigma-1 receptors in the brain.

There is ample evidence suggesting that Sigma-1 receptors are capable of mediating protection against cerebral ischemic stroke. For example, the high affinity Sigma-1-selective ligand PPBP decreases transient focal ischemia-induced brain injury in rats, cats, and mice (Takahashi et al., 1995, 1996; Goyagi et al., 2001). Given that many studies have demonstrated that PPBP protects against brain ischemia, we saw no reason to repeat these results in the current study. Furthermore, our results for haloperidol are consistent with the finding that PPBP reduces infarct volume in the rat tMCAO model by a mechanism that does not require altering dopamine levels (Goyagi et al., 2003). This is relevant as PPBP, like haloperidol, binds with low submicromolar affinity to cloned D2 receptors (data not shown). Although it is unclear whether PPBP acts as an agonist or antagonist of Sigma-1 receptors, the present study using the prototypical Sigma-1 antagonist haloperidol suggests that neuroprotection is mediated by antagonizing the Sigma-1 receptor.

The experimental results presented here indicate that low levels of the antipsychotic drug haloperidol significantly protect against oxidative stress-induced cell death *in vitro* and *in vivo.* The likely mode of drug action at the low level of haloperidol investigated here is likely due to antagonism of the Sigma-1 receptor. Given the long history of haloperidol use

in humans to treat other indications and the lack of adequate effective treatments for acute ischemic stroke, the possible value added potential of acutely administered haloperidol or associated butyrophenone antipsychotics as protectants against ischemic cerebral stroke and other types of brain injury with an ischemic component warrants further investigation.

4. Experimental procedures

4.1. In vitro determination of HT-22 cell viability: calcein AM assay

Cell survival following a glutamate insult in the presence or absence of protective agents was determined with the vital dye calcein acetoxymethyl (AM) (Molecular Probes, Eugene, OR) as described previously (Bozyczko-Coyne et al., 1993). This assay takes advantage of the fact that calcein is strongly fluorescent and cell impermeant, while its acetoxymethylester (AM) derivative is both cell-permeable and non-fluorescent. Once the calcein AM is inside the cell, the AM portion is cleaved by endogenous non-specific esterases. Consequently, only viable cells exhibit fluorescence.

HT-22 cells were obtained as a gift from David Schubert, Salk Institute, San Diego, CA. Cells (passages 18–25) were seeded into Costar 96-well plates (Corning, NY) at a density of 5000 cells/well and then maintained in Dulbecco's modified Eagle's (DMEM) media (GIBCO, Gaithersburg, PA) supplemented with 10% charcoal-stripped fetal bovine serum (HyClone, Logan, UT) and 20 μg/ml gentamycin (Sigma, St. Louis, MO) under standard cell culture conditions (5% CO_2, 95% air, 37 °C). Compounds were administered coincident with glutamate insult then approximately 16 h. Media were then removed and cells were incubated in a 1 μM solution of calcein AM in PBS. After 15 min, the calcein AM solution was removed and cell viability was determined by measuring the fluorescence (excitation 485 nm, emission 530 nm) on a fluorescence FL600 microplate reader (Biotek, Winooski, VT).

4.2. Determination of compound affinity for the cloned Sigma-1 receptor: creation of a Sigma-1 receptor stable cell line and [^{3}H]-(+)-pentazocine competition binding

A cloned Sigma-1 receptor (GenBank accession no. BC004899) was stably expressed in MCF-7 cells, because this cell line has no specific binding for [^{3}H]-(+)-pentazocine, the radioligand used to detect Sigma-1 receptors (Vilner et al., 1995; Seth et al., 1998; Yamamoto et al., 1999; Shamsul Ola et al., 2002). The full length Sigma-1 receptor was subcloned into a pcDNA3.1 (Invitrogen, CA) plasmid construct and then transfected into MCF-7 cells using a calcium phosphate ($CaPO_4$) precipitation kit (Invitrogen, CA) as described previously (Kortagere et al., 2004). Briefly, 20 μg of purified plasmid DNA was mixed with a final volume of 1 ml $CaPO_4$/HEPES solution and the resulting precipitate was added drop wise to a 150 cm^2 plate containing MCF-7 cells at 5–10% confluence. The following day, the media were removed by aspiration and replaced with fresh media containing the appropriate concentration of selection agent (e.g., 2 mg/ml geneticin (G418)). Cells were grown under constant G418 selection and individual clones were selected once colonies start to appear. The expression levels of the Sigma-1 receptor in individual clones were determined by [^{3}H] (+)-pentazocine (Perkin-Elmer, specific activity 36.6 Ci/mmol) saturation isotherm binding using the standard rapid filtration techniques as previously

described (Kortagere et al., 2004). Briefly, cells were dislodged by a 10 min incubation in Earle's balanced saline solution (EBSS) lacking Ca2+ and Mg2+ and supplemented with 5 mM EDTA. The cell solution was diluted in four volumes of EBSS and then pelleted by centrifugation at 900×*g* for 10 min. Following centrifugation, the supernatant was decanted and the cell pellet was lysed in lysis buffer (5 mM Tris, 5 mM $MgCl_2$, pH 7.4). The lysate was glass-glass homogenized and the membranes were isolated by centrifugation at 35,000×*g* for 60 min. The membrane pellet was re-suspended in binding buffer (50 mM Tris, pH 7.4, at 25 °C) and washed by re-centrifugation. The washed membrane pellet was re-suspended by light homogenization in Sigma-1 receptor binding buffer (50 mM Tris, pH 8.0 at 37 °C) immediately before use in the radioligand binding assays. Non-specific binding was defined by 5 μM haloperidol or 5 μM BD1063. The binding reaction was allowed 3 h to reach equilibrium at 37 °C with moderate shaking prior to rapid filtration through GF/C filters pretreated with 0.3% polyethyleneimine. The wash buffer consisted of ice-cold binding buffer (10 mM Tris, pH 8.0, at 2 °C). Radioactivity bound to the filters was quantified by scintillation spectroscopy. Membrane protein concentration was determined using the bicinchonic acid (BCA) protein reagent (Pierce, IL) and a bovine serum albumin standard curve exactly as described previously (Kortagere et al., 2004). The same binding conditions and cell line were utilized to determine the affinity (K_i) of compounds for the Sigma-1 receptor employing competition binding assays, with the exception that increasing concentrations of the test compound were used to compete with a fixed concentration of [^{3}H]-(+)-pentazocine (1 nM). The inhibition constant (K_i) values were calculated from IC_{50} values using the Cheng-Prusoff equation: $K_i = IC_{50}/(1+[ligand]/K_D)$ (Cheng and Prusoff, 1973). A 95% confidence interval was used for all curve fitting procedures and for comparing different curve fitting models. The statistical measures of curve fitting were the *F*-test, the run test, and a correlation coefficient (r^2). All analyses utilized Graphpad's Prism software (version 4.0).

4.3. In vivo protection against transient ischemic stroke: ovariectomy, transient Middle Cerebral Artery Occlusion (tMCAO) stroke model and determination of ischemic lesion volume

Four to six week old female Sprague–Dawley rats were purchased from Charles Rivers (Wilmington, MA) and maintained in our animal facility in a temperature-controlled room (22–25 °C) with 12-h dark-light cycles. All rats had free access to laboratory chow and tap water. Ovariectomy was performed using a dorsal approach as described previously (Simpkins et al., 1997). A minimum of 14 days after ovariectomy, tMCAO, was performed as originally described by Longa et al. (1989) using a reversible intraluminal filament occlusion technique under conditions of normoxia, normocarbia, and normothermia. Briefly, the internal carotid artery (ICA) of anesthetized animals was exposed, and a 3-0 monofilament nylon suture was introduced into a puncture and gently advanced into the lumen to the distal internal carotid artery (ICA) until proper resistance was felt. After 1 h, the suture was withdrawn and the ICA puncture was cauterized. A single acute dose of neuroprotective compound was administered via subcutaneous injection coincident with the initiation of the occlusion. A subcutaneous route was selected, because haloperidol was administered concurrent with the onset of tMCAO and intravenous administration in a manner that would have substantially enhanced brain distribution (e.g., via internal carotid

artery) would have required an additional surgery. Furthermore, this would not have been clinically relevant and the kinetics of haloperidol after subcutaneous administration is sufficient to achieve a rapid brain distribution.

At 24 h after reperfusion, stroke severity and the potential neuroprotective effects of compounds were assessed as infarction volume utilizing 2,3,5-triphenyltetrazolium chloride (TTC) histochemistry coupled with quantitative image analysis as described previously (Liu et al., 2002). Briefly, rats were decapitated, and their harvested brains were rinsed in normal saline, placed in a brain matrix, and cut into seven coronal slices 3, 5, 7, 9, 11, and 13 mm posterior to the olfactory bulb. Brain slices were incubated at 37 °C for 30 min with mild agitation in a 2% solution of TTC prior to fixation in a 10% formalin solution. TTC-stained slices were then photographed with a digital camera and ischemic lesion volume was quantified with a color image analysis system (Image-Pro Plus 4.1 Media Cybernetics). TTC staining allows demarcation of damaged or infarcted tissue (white color) from normal, viable cerebral tissue (red color), because only viable (i.e., respiring) cells are capable of forming the red-colored formazan product, which is created via the reduction of TTC by mitochondrial dehydrogenase enzymes. These studies have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

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Abbreviations

tMCAO	transient middle cerebral artery occlusion
PPBP	4-phenyl-1-(4-phenylbutyl) piperidine
NMDA	*N*-methyl-D-aspartate

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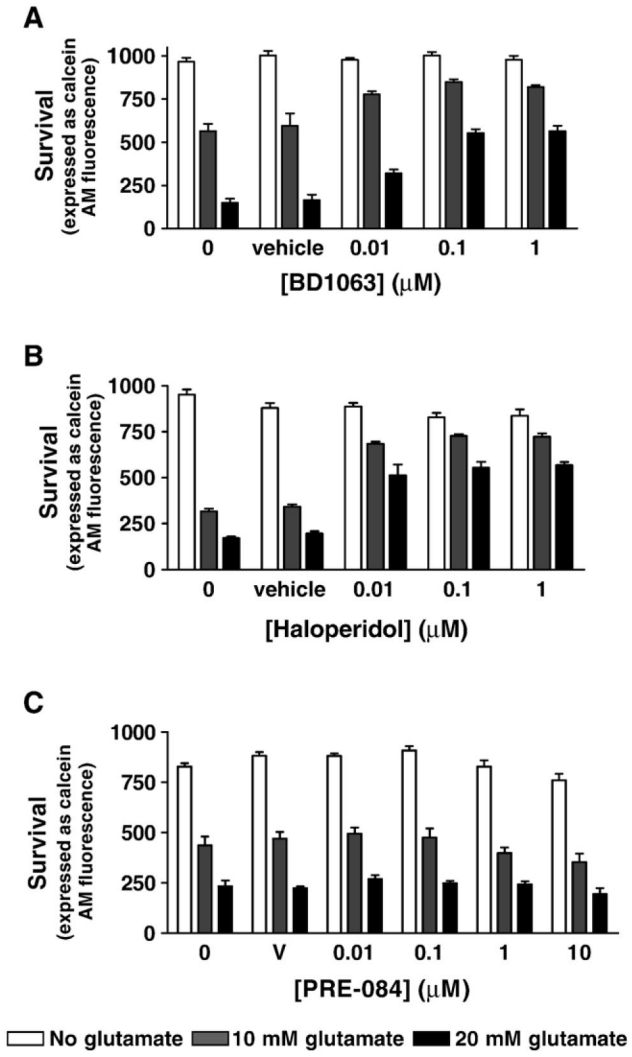

Fig. 1.

(A and B) The Sigma-1 receptor antagonists BD1063 and haloperidol protect against glutamate-induced oxidative stress cell death in an *in vitro* HT-22 cell model. Increasing concentrations of glutamate result in higher levels of oxidative stress leading to higher levels of cell death. Cell survival is measured with the fluorescent vital dye calcein AM. (C) The Sigma-1 receptor-selective agonist PRE-084 provides no protection.

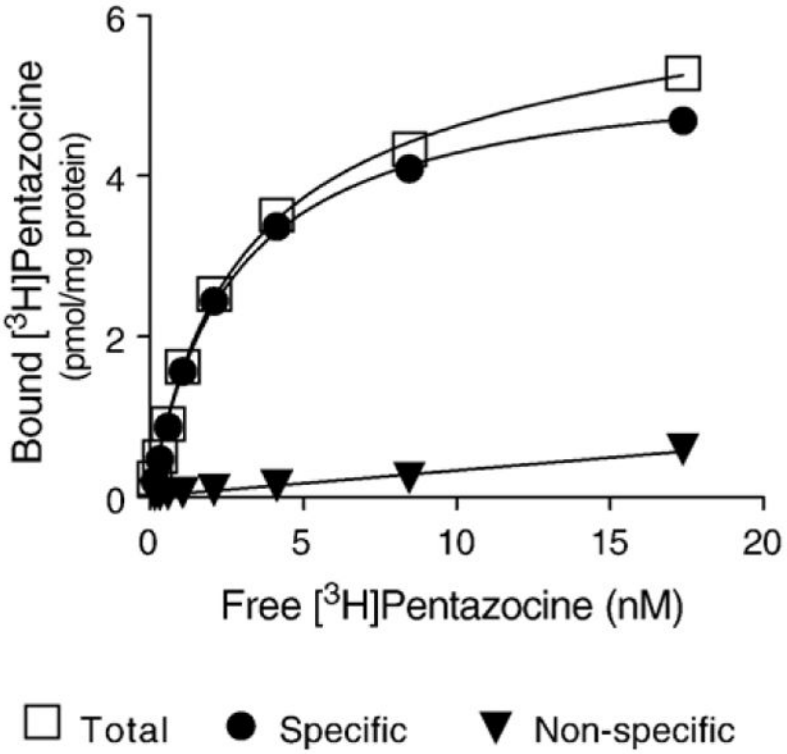

Fig. 2.

Representative example of [^{3}H]-(+)-pentazocine saturation isotherm binding to purified membranes from the hippocampal HT-22 cell line. The calculated affinity and receptor density values, expressed as the geometric means of three separate experiments (n=3) and their associated standard deviations, are K_D =4.2±1.7 nM and B_{max} =5.0± 0.83 pmol/mg membrane protein, respectively.

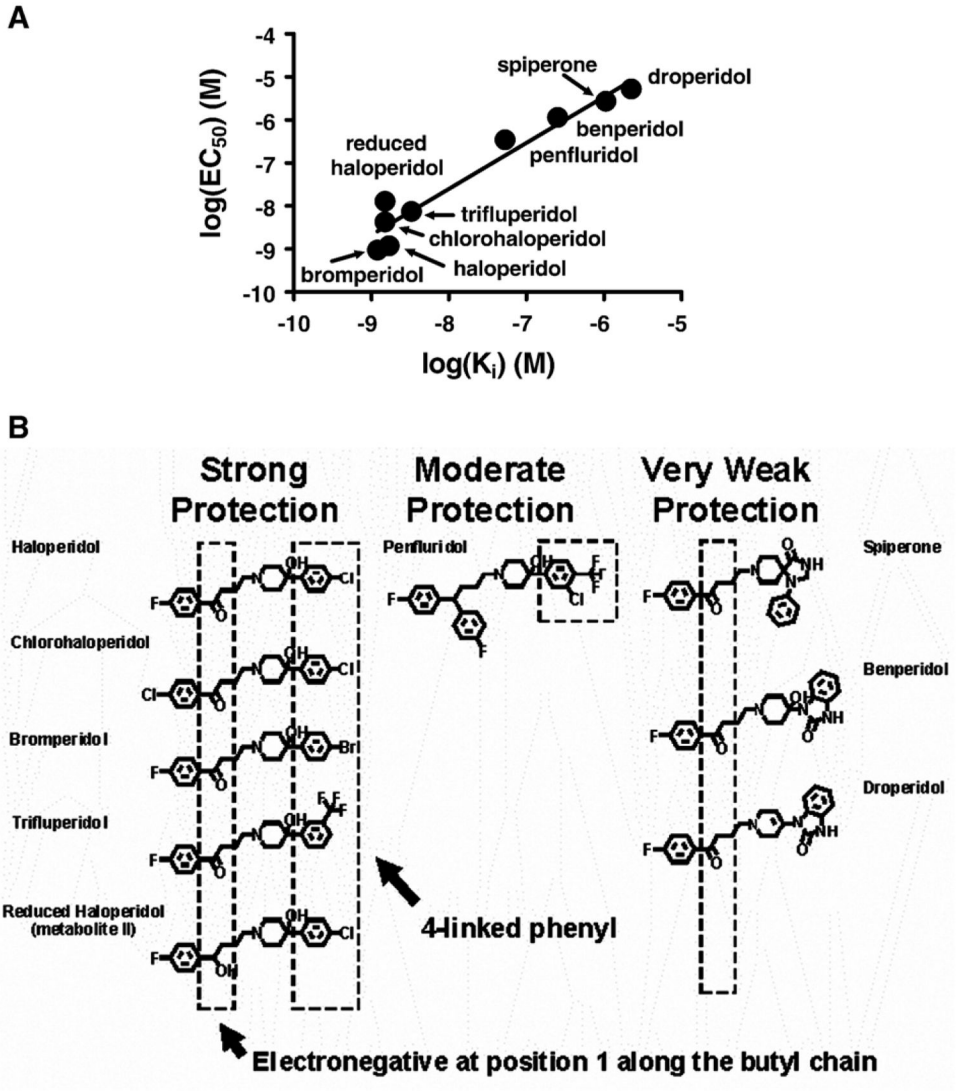

Fig. 3.

Correlation analysis of the potency of *in vitro* protection and affinity for the cloned Sigma-1 receptor, and structure–protection relationships of butyrophenones. Potency values were determined using 20 mM extracellular glutamate to induced oxidative stress in HT-22 cells. (A) Correlation for butyrophenone antipsychotics. (B) Chemical structures of butyrophenone antipsychotics. Substructural features important for the protective effect are boxed with dashed lines. The presence of a 4-linked phenyl is more critical for activity than having an electronegative moiety at position one along the butyl chain, and a high potency effect requires the presence of both substructural features. Strong protection is defined as EC_{50} <20 nM, moderate protection as 20 nM>EC_{50} <500 nM, and very weak protection as EC_{50} >500 nM, respectively. The correlation between potency of neuroprotection and affinity for the cloned Sigma-1 receptor is highly significant (P<0.001), Pearson's correlation coefficient r^2=0.942. The efficacy of the strongly protective compounds, calculated as a percentage of the survival in the untreated (top) minus survival after treatment with 20 mM glutamate (bottom), ranged from 55±3.0% to 67±9.8%.

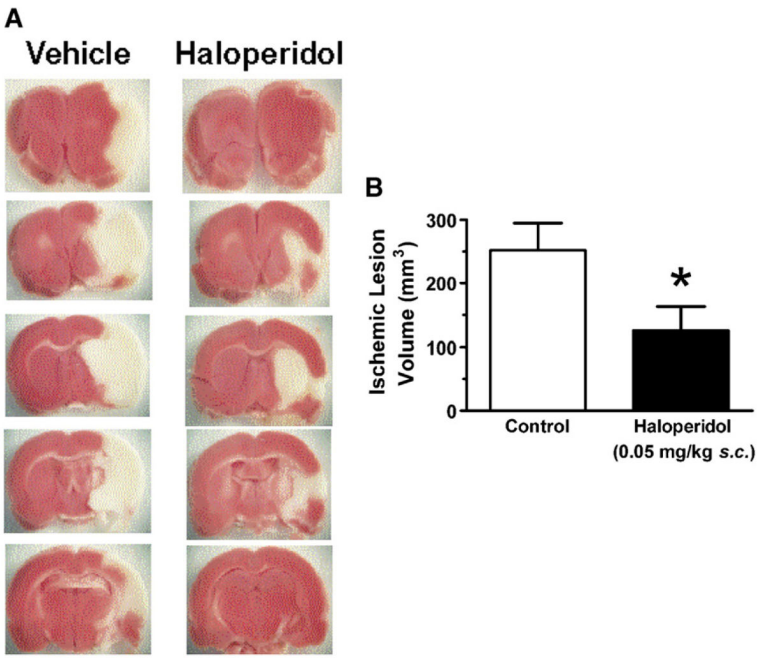

Fig. 4.

Haloperidol protects *in vivo* against tMCAO brain injury. (A) Representative triphenyltetrazolium-stained coronal brain slices (top to bottom corresponds to anterior to posterior) from tMCAO (hemi)stroked ovariectomized female Sprague–Dawley rats and protection by an acute low dose of haloperidol (0.05 mg/kg s.c.). Dead tissue appears white while living tissue appears red. (B) Averaged protection data assessed as ischemic lesion volume. **P*<0.05, Vehicle control treated vs. haloperidol treated. The *n*=6 animals for the vehicle control group and *n*=7 for the haloperidol-treated group. Error bars in this graph represent the standard deviations.

Table 1

Structure–activity and structure–affinity relationships for butyrophenones *in vitro*

Drug/compound name	Affinity for the cloned Sigma-1 receptor (K_i ±SEM, nM)	Potency (EC_{50} ±SEM nM) and *Efficacy±SEM*	Comments
Haloperidol	1.7±0.46	1.2±0.4 *65±2.5*	Typical antipsychotic; butyrophenone structure
Trifluperidol	3.3±0.06	7.5±6.3 *55±3.0*	Haloperidol congener
Chlorohaloperidol	1.5±0.36	4.3±1.7 *64±3.4*	Haloperidol congener
Bromperidol	1.2±0.21	0.95±0.82 *67±9.8*	Haloperidol congener
Penfluridol	53±15	350±246 *80±20*	Pimozide-like; a diphenylbutylpiperidine
Haloperidol metabolite II (reduced haloperidol)	1.5±0.47	12.9±12.2 *58±5.6*	Similar affinity as haloperidol for Sigma-1, but ~350-fold less affinity for D2 receptors
Spiperone	1054±334	2737±714 *100*	Typical antipsychotic; butyrophenone structure
Droperidol	2240±499	5271±2230 *100*	Spiperone-like
Benperidol	252±42	1157±498 *66±23*	Spiperone-like

Activity is defined as protection against glutamate-induced oxidative stress cell death in an *in vitro* HT-22 cell model. Increasing concentrations of glutamate result in higher levels of oxidative stress leading to higher levels of cell death. Cell survival is measured with the fluorescent vital dye calcein AM. Affinity (K_i) values for the compounds listed were measured by [^{3}H]-(+)-pentazocine competition binding assays using cloned Sigma-1 receptors in an unambiguous assay system (Lee et al., in press).