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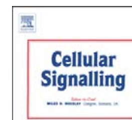

Haloperidol disrupts Akt signalling to reveal a phosphorylation-dependent regulation of pro-apoptotic Bcl-XS function

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ABSTRACT

The antipsychotic drug haloperidol is still used to treat psychosis and "agitation", often with devastating consequences, particularly in geriatric and pre-demented patients. Cytotoxicity induced by haloperidol has been associated with induction of Bcl-XS, a pro-apoptotic member of the Bcl-2 family, as well as with modulation of the Akt pro-survival pathway. Using preneuronal PC12 and primary neuronal cultures, we show that haloperidol inactivates Akt. This induces the dephosphorylation of serine residues in Bcl-XS and promotes its association with the mitochondrial voltage-dependent anion channel (VDAC), as well as with cytochrome *c*- and caspase-3-dependent events. These events are sensitive to expression of constitutively active Akt. Mutation of Serine106 (Ser106), which is flanked by a putative Akt motif, hinders the association of the Bcl-XS protein with Akt, but promotes its association with VDAC. The dephosphorylation mimic, Bcl-XS(Ser106Ala), induces caspase-dependent PC12 and neuronal cell apoptosis. In contrast, Bcl-XS(Ser106Ala) induces a significant loss of VDAC expression, and cytochrome *c*- and caspase-independent toxicity in the non-neuronal HEK293A cells. We link haloperidol and Akt to Bcl-XS-sensitive toxicity *via* cell line-dependent mitochondrial events centering on VDAC. This clearly mitigates the chronic use of haloperidol in neuropsychiatric populations, but supports its use as a potential acute therapeutic in cancer, where apoptosis is desirable.
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1. Introduction

The phosphatidylinositol 3′-kinase (PI3K)/Akt pathway promotes survival *via* phosphorylation-dependent inactivation of pro-apoptotic effectors including members of the Bcl-2 family of proteins (see [1] for review; [2]). Bcl-2-related proteins contain structural features referred to as Bcl-2 homology (BH) domains. The best characterized pro-apoptotic Bcl-2 family member, Bax, has the obligatory BH3 domain in addition to membrane-spanning BH1 and BH2 domains that contribute to channel formation in synthetic membranes and lipid vesicles. In contrast, pro-apoptotic Bcl-XS, devoid of BH1 and BH2 domains and lacking the potential to form membrane-spanning channels, must act through a different mechanism. Bcl-XS does have a BH3 domain as well as a BH4 domain (thought to allow for protein–protein interactions outside the Bcl-2 family), which is a unique combination among the Bcl-2 family members [3]. The modulation of cytochrome *c* release from the mitochondria might reflect channel formation by Bcl-2 homologue oligomers, for example, Bax [4], as well as by hybrid channels formed by associating with a pore complex centered on the voltage-dependent anion channel (VDAC) [5–7].

Bcl-2-related proteins are crucial in regulating apoptosis and often exert their pro- or anti-apoptotic effects following some means of post-translational modification; this can involve, for example, cleavage (*e.g.* of Bcl-2, Bid, or Bim) or phosphorylation. Bcl-2-Thr56 can be phosphorylated during mitosis by CDK [8] and JNK [9]. This phosphorylation of Bcl-2 might disable its anti-apoptotic function [9,10], yet multisite phosphorylation might actually stabilize Bcl-2 and promote its anti-apoptotic function [11]. JNK phosphorylates Bcl-XL within its activation loop on Ser62 [12] and possibly on threonine residues [13]. Akt-mediated phosphorylation of Bax-Ser184 sequesters it in the cytoplasm away from mitochondrial targets [2]. Multisite phosphorylation of Bad-Ser112 by ERK and Ser136 by Akt [14,15], on Ser155 (by PKA, but only if Ser136 is phosphorylated: [16]) and on Ser170 [17] promotes, in part, its association with the cytoplasmic 14-3-3 proteins and suggests a need for tight control of this highly effective pro-apoptotic protein. Akt phosphorylates Bim-Ser87, which promotes its binding with 14-3-3 proteins [18]. The phosphoregulation of pro-apoptotic Bcl-XS has as yet to be assessed, although work on its anti-apoptotic variant Bcl-XL suggests that multisite phosphoregulation is highly probable.

We have demonstrated that apoptosis induced in preneuronal PC12 and neuroblastoma N2a cultures by the antipsychotic drug haloperidol (HAL) is dependent on the sigma2 (σ2) receptor, rather

Abbreviations: HAL, haloperidol; DTG, 1,3-di-*o*-tolylguanidine; SUL, sulpiride; PARP, poly(ADP-ribose) polymerase; VDAC, voltage-dependent anion channel.

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than the anticipated dopamine D2 receptor, and on induction and mitochondrial accumulation of Bcl-XS [19,20]. This is a p53/Bax-independent process [20]. Furthermore, we have clearly demonstrated that HAL can inactivate Akt in PC12 cells by disruption of the PI3K/Akt cascade at the level of PDK1, the upstream activator of Akt [21]. Others have also linked PI3K/Akt function to the pharmacology of antipsychotics, including HAL, and with schizophrenia [22–24].

We hypothesized that HAL-mediated toxicity results from an Akt-sensitive, Bcl-XS-mediated event. We chose to use HAL, not only because of its continued use in the clinical setting, but also because it is a useful tool for the study of apoptosis and cytotoxicity. We tested our hypothesis in the PC12 cell line which can undergo apoptosis *via* distinct Bcl-XS- and Bax-mediated cascades [25,26], and as such is a good *in vitro* model for examining the role of signalling cascades on these two Bcl-2-related proteins. Our observations in PC12 cells were compared and contrasted with those obtained for primary cortical neurons and for the non-neuronal HEK293A cell line.

2. Experimental/materials and methods

2.1. Chemicals and antibodies

Antibodies recognizing Bcl-XS/L, caspase-9 [recognizes both mature (48 kDa) and cleaved (35 kDa) proteins] and PARP were obtained from Santa Cruz Biotechnology. The VDAC antibody was from Calbiochem Anti-Bcl-X and anti-cytochrome *c* was obtained from BD Transduction Laboratories, while anti-β-actin and anti-phosphoserine, protease inhibitor cocktail, haloperidol, sulpiride and DTG were from Sigma-Aldrich, Inc. Anti-Akt and anti-caspase-3 [recognizes the cleaved (17/19 kDa) species], and the PI3K inhibitor LY294002 were from Cell Signaling Technology. IgG-HRP conjugates were from Cedarlane Laboratories. The cell permeable caspase-3 inhibitor Z-DEVD-fmk was from R&D Systems.

2.2. Immortalized cell and primary cell cultures

The PC12 rat preneuronal cell line (CRL-1721) and the HEK293 human embryonic kidney cell line (CRL-1573) were purchased from ATCC and were maintained according to their specifications.

Fetal rat (E18) primary neuronal cultures were prepared as described [27]. Timed-pregnant Sprague–Dawley rats were obtained from Charles River (St. Constant, QC) and maintained according to guidelines set by the University of Saskatchewan Animal Care Committee and the Canadian Council for Animal Care. Treatments of primary cells were performed on day 7 of culture.

2.3. Plasmids, cDNA mutagenesis and transfection

CS2+myr-Akt expresses a membrane-directed, constitutively active, *N*-myristoylated full-length mouse Akt1 (myr-Akt: a gift from Dr. A.B. Vojtek, University of Michigan, Ann Arbor). Wildtype Bcl-XS was amplified from PC12 cDNA and was subcloned into the pCMV expression vector (Invitrogen Life Technologies) or in-frame with an N-terminal triple-FLAG (a gift from Dr. D.H. Anderson, University of Saskatchewan). Bcl-XS serine-to-alanine substitutions were generated with the QuikChange® kit (Stratagene). Cells in log phase were transfected with plasmid DNA (1–2 μg/well on a 24-well plate; seeded at 5×10^5 cells/well) using LipofectAmine Plus (Invitrogen Life Technologies). Expression of eGFP revealed a transfection efficiency of 35% and 60% in PC12 and HEK cells, respectively.

2.4. Immunodetection and subcellular fractionation

Standard SDS-PAGE Western blot conditions were used to detect expression of targeted proteins in total cell lysates (precleared; 5000 ×g, 10 min, 4 °C; 20–30 μg/lane) or in immunoprecipitates (300–500 μg: precleared lysates using non-specific mouse or rabbit IgG) isolated with selected antibodies and precipitated with protein-A/G Sepharose. Detection relied on enhanced chemiluminescence (Pierce) and depicted blots are representative of 2–3 individual experiments.

Subcellular fractionation was achieved using differential centrifugation [21]. Briefly, lysates were centrifuged (900 ×g, 10 min, 4 °C) and the resulting supernatant was centrifuged (18,000 ×g, 40 min, 4 °C) to yield a mitochondrial-enriched pellet and a supernatant that represented the cytosolic [soluble] fraction.

2.5. Cell viability assays

Hoechst 33258 staining ($n\geq3$) in primary cells was used to reveal chromatin condensation, a nuclear characteristic of apoptosis. Conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium] to formazan crystals reflects mitochondrial function and cell viability in PC12 cells, as previously validated [20]. Experimental means are based on ≥6 individual experiments, each of which is represented by 4–6 replicates per test group.

2.6. Statistical analysis

Significance (set at $P<0.05$) was assessed by ANOVA and Bonferroni's Multiple Comparison Test (*post hoc*).

3. Results

3.1. HAL-induced apoptotic primary neuronal cell death is not dependent on the dopamine D2 receptor system

HAL is a ligand for the dopamine D2 receptor as well as for σ receptors in functional and binding assays. A role for the dopamine D2 receptor in the current model is precluded by the inability of the D2 receptor antagonist sulpiride (SUL), which lacks σ receptor binding affinity [28], to impact primary neuronal cell viability as measured by MTT conversion (Fig. 1a). HAL is toxic to these cells, with an LC50 value of 57±16 μM (*note*, LC50 is the [lethal] concentration required to kill 50% of the test culture) (Fig. 1a). Co-treatment with either SUL or with the highly-charged (and cell impermeable) σ ligand DTG does not impact the cytotoxicity of HAL (Fig. 1b).

In primary cortical neurons, HAL induces pro-apoptotic Bcl-XS (but not pro-apoptotic Bax) expression in a time-dependent manner (Fig. 1c). The time-dependent HAL-induced inactivation of Akt (as indicated by its dephosphorylation, particularly on Thr308: Fig. 1c) precedes the appearance of pro-apoptotic events such as the cleavage of caspase-9 to the active p35 fragment as well as the cleavage of the executioner caspase-3 to the active p17 species (Fig. 1c). Apoptosis and the involvement of caspase-3 are further corroborated by concurrent cleavage of PARP to the p85 species (a caspase-3-dependent process: [29]). Staining with Hoechst 33258 reveals an increase in the number of cells exhibiting chromatin condensation (a hallmark of the terminal stages of apoptosis; Fig. 1d) in HAL-treated cultures. The number of apoptotic nuclei in cells treated with DTG or SUL is similar to that in control cultures (Fig. 1d). HAL (but not DTG) induces the expression of Bcl-XS and the dephosphorylation of Akt (Fig. 1e), and the activation of both caspase-9 and -3, as demonstrated by the increase in their respective active cleavage products (Fig. 1f).

3.2. HAL-induced apoptosis is sensitive to Akt and dependent on caspase-3 in primary neuronal cells

Expression of a myristoylated, constitutively active Akt (myr-Akt; Fig. 2c) reverses the chromatin condensation associated with HAL (a representative example is depicted in Fig. 2a and total experimental data are quantitated in Fig. 2b). Treatment with the PI3K inhibitor LY294002 inhibits Akt phosphorylation, as expected (Fig. 2d), and is toxic

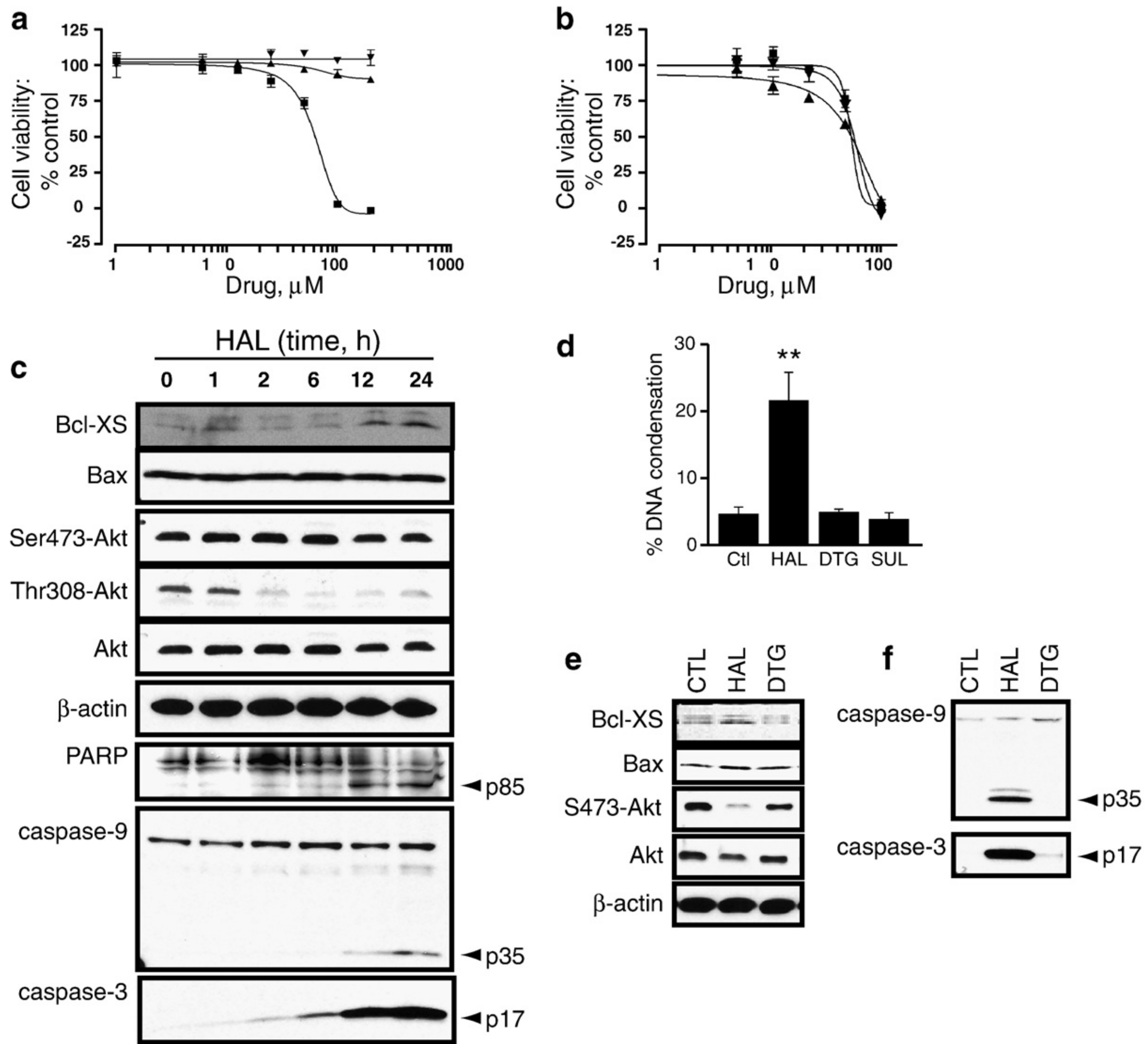

Fig. 1. HAL toxicity in primary neuronal cultures occurs *via* the σ2 receptor system and coincides with the loss of Akt activity. (a) Primary cortical neurons were treated for 24 h with increasing concentrations of haloperidol (HAL: ■), sulpiride (SUL: ▼) or DTG (▲) and cell viability was determined by the MTT conversion assay ($n \geq 3$). (b) Cortical neurons were treated with either HAL alone (■) or co-treated with HAL+SUL (▼) or HAL+DTG (▲) (all drugs at 100 μM, 24 h); cell viability was determined by MTT conversion ($n \geq 3$). (c) Cortical cultures were treated with HAL (100 μM, 1–24 h) and precleared protein extracts were analyzed by Western blot for the expression of pro-apoptotic Bcl-XS and Bax, for Akt phosphorylation [on Serine473 (Ser473) and Threonine308 (Thr308)], or for the p85 PARP fragment, or the p35 caspase-9 and p17 caspase-3 fragments. (d) Chromatin condensation (a hallmark of cells in the final stages of apoptosis visualized using Hoechst nuclear staining) was used to quantitate apoptotic nuclei in HAL-, DTG- and SUL-treated neuronal cultures ($F(3,11)=14.19$; **: $P<0.01$). (e, f) Precleared protein extracts from HAL- and DTG-treated (100 μM, 24 h) cortical cultures were compared for similar protein targets as in (b) above. β-Actin was included as a loading control.

to neuronal cells even in the absence of HAL (Fig. 2e). Treatment with the caspase-3 inhibitor DEVD reverses HAL-induced cytotoxicity (Fig. 2f). As expected, DEVD does not interfere with the actual cleavage of caspase-3, but clearly inhibits its activity as demonstrated by the reduction in HAL-induced PARP cleavage to the p85 fragment (Fig. 2g). These data confirm that HAL-induced apoptosis is a caspase-3-dependent event. LY294002 induces caspase-3 cleavage as well as PARP cleavage on its own and does not add to the effect of HAL (Fig. 2g). Thus, the apoptotic component of HAL cytotoxicity is mediated predominantly by the inhibition of the PI3K/Akt pathway.

3.3. HAL-induced toxicity in PC12 cells is Akt-sensitive and cytochrome c-dependent

HAL treatment results in the dephosphorylation of Akt in PC12 cells (Fig. 3) as it does in our primary neuronal cultures. Furthermore, in PC12 cells this corresponds with pro-apoptotic events including the mitochondrial accumulation of Bcl-XS, the release of cytochrome *c* (cyto *c*) to the cytoplasm and cleavage of caspase-9 (Fig. 3).

The constitutively active myr-Akt does not prevent HAL-induced Bcl-XS expression, but does reduce the detection of Bcl-XS in the mitochondria (Fig. 4a). This coincides with the retention of cytochrome *c* in the mitochondria and the inhibition of PARP cleavage. The expression of myr-Akt diminishes the cytotoxicity induced by HAL (Fig. 4b) and induces an upward shift in the response curve to HAL (Fig. 4c). The change in slope on a double-reciprocal plot (Fig. 4c, insert), which reveals a diminished potency in the presence of Akt, *e.g.* the HAL+Akt line is higher than the HAL (alone) line, and an unaltered affinity, *e.g.* the two lines intersect, indicating that the effect of myr-Akt on HAL represents non-competitive functional antagonism.

3.4. HAL promotes a phosphoseryl-sensitive complex between Bcl-XS and VDAC

The interaction of Bcl-2 family members with a mitochondrial pore complex that centers on the channel-forming protein VDAC is a well known means of modulating cytochrome *c* release. We demonstrate that HAL promotes the detection of VDAC in anti-Bcl-XS immunoprecipitates

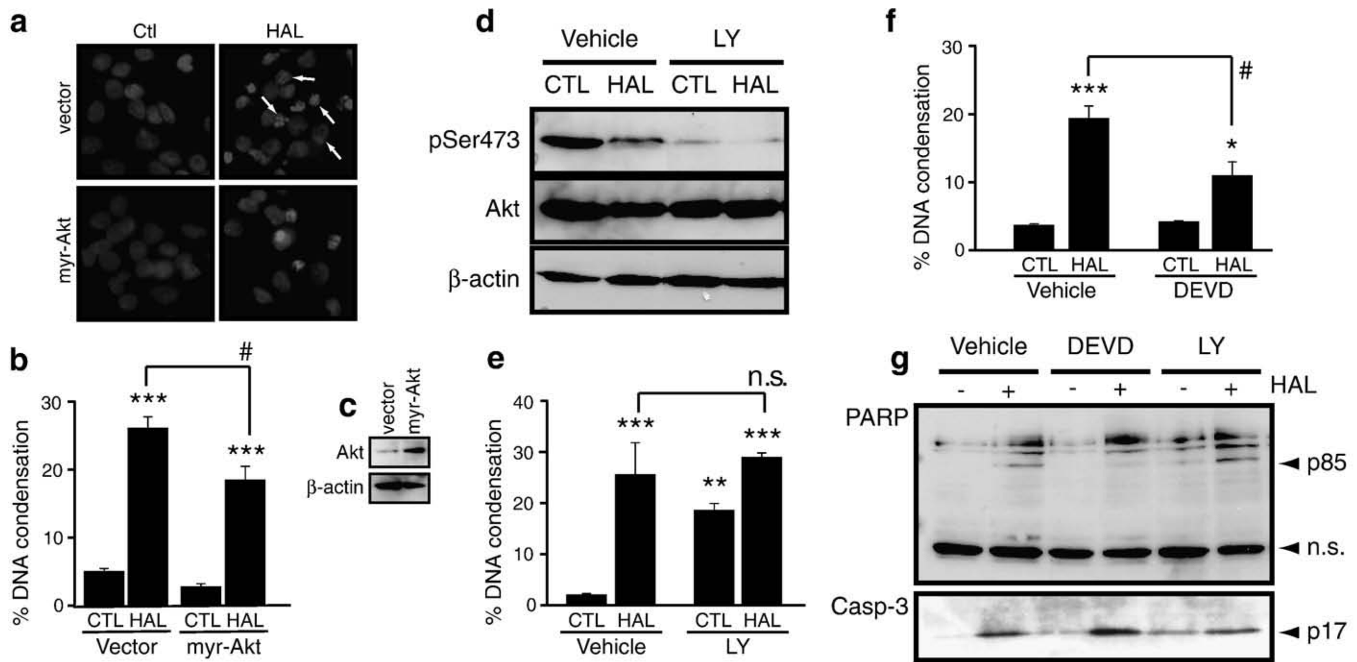

Fig. 2. HAL toxicity in primary neuronal cultures occurs *via* the σ2 receptor system and coincides with the loss of Akt activity. Primary cortical neurons were transfected with the CS2+ vector (vector) or with the constitutively active, myristoylated Akt (myr-Akt) expression plasmid (as shown in panel c). (a) A representative field from one culture/experiment demonstrates (arrows) the condensed chromatin (visualized using Hoechst nuclear staining). (b) Quantitation of the effect of overexpressed myr-Akt on HAL-induced chromatin condensation ($F(3,11)=83.43$; ***: $P<0.001$ versus the respective controls; #: $P<0.05$ between the indicated groups). (d) Neuronal cultures were pretreated with the PI3K/Akt inhibitor LY294002 (LY: 25 µM, 30 min) and then treated with vehicle control (CTL) or HAL (100 µM, 24 h). Corresponding protein extracts were examined by Western blot for Akt phosphorylation. (e) Treatment with LY induces chromatin condensation whether used on its own or in conjunction with HAL ($F(3,11)=23.30$; **: $P<0.01$ and ***: $P<0.001$ versus vehicle-treated control (CTL); n.s. not significant between indicated groups). (f) Treatment with the cell permeable caspase-3 inhibitor DEVD-fmk (DEVD) reverses HAL-induced chromatin condensation ($F(3,14)=23.14$; *: $P<0.05$ and ***: $P<0.001$ versus vehicle-treated control (CTL); #: $P<0.05$ between indicated groups). (g) Protein extracts from HAL-treated cultures co-treated with either DEVD-fmk (DEVD) or LY were examined by Western blot analysis for PARP cleavage to the p85 fragment or for caspase-3 (Casp-3) cleavage to the active p17 fragment.

and that this is reversed by overexpression of myr-Akt (Fig. 4d). Furthermore, the phosphoseryl status of Bcl-XS is decreased by HAL and recuperated by concurrent expression of myr-Akt (Fig. 4d). This suggests that an Akt-dependent change in Bcl-XS phosphorylation could predispose to its interaction with VDAC. The phosphorylation of Bcl-XS on tyrosine and threonine is negligible and is not altered by HAL (data not shown).

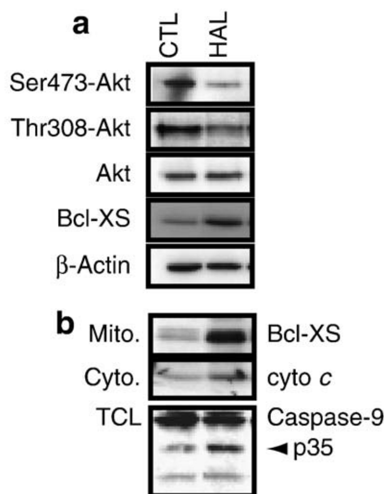

Fig. 3. HAL induces Bcl-XS expression and apoptotic events in PC12 cells. Precleared protein extracts from PC12 cells treated with HAL (125 µM, 24 h) were analyzed by Western blot for (a) Akt phosphorylation on Serine473 (Ser473) and Threonine308 (Thr308) as well as for Bcl-XS expression. (b) Similarly-treated PC12 cultures were used for fractionation studies to determine the subcellular localization [mitochondria: Mito.; cytoplasm: Cyto.] of Bcl-XS and cytochrome *c* (cyto *c*) or for caspase-9 cleavage to the p35 fragment in total cell lysates (TCL).

3.5. The dephosphorylation of pro-apoptotic Bad is also sensitive to activation of Akt

Treatment with HAL results in the dephosphorylation of pro-apoptotic Bad on Ser136 (Fig. 5a), a residue known to be targeted by Akt and known to regulate Bad toxicity [15,30]. The overexpression of activated Akt (myr-Akt) also reverses the HAL-induced dephosphorylation of Bad (Fig. 5b), as it did the dephosphorylation of Bcl-XS (*see* Fig. 4d, above). While we have not extensively examined the role of Bad in our apoptotic cascade, these data are included to support our hypothesis that HAL induces a critical [Akt-mediated] phosphorylation-dependent event and to confirm that this HAL does not alter Bax (Fig. 5a), as previously reported [20,25,31].

3.6. Specific serine residues in Bcl-XS dictate its toxicity

Targeted alanine (Ala; A) substitution of selected serine (Ser; S) residues was used to determine their respective contribution to Bcl-XS function. We chose Ser62 because it is placed within the loop region and is targeted for phosphorylation-induced inactivation of the *bcl-X* splice variant Bcl-XL (and analogous to Ser70 in Bcl-2) [9,12]. We also targeted Ser106, which resides in a non-canonical Akt motif as well as Ser165, which lies within the C-terminal hydrophobic “anchor” region and is ostensibly analogous to the Bax-Ser184 residue required for membrane localization of Bax [32].

In our experience, PC12 cells do not express toxic proteins very readily; perhaps this explains the consistently lower expression of Bcl-XS mutants (*cf.* the expression of wildtype Bcl-XS) in these cells (Fig. 6a). Of the three mutants, only transiently expressed Bcl-XS(S106A) and -(S165A) decrease PC12 cell viability (Fig. 6a). In contrast, all three Bcl-XS mutant proteins reduce the viability of the non-neuronal HEK293A cell line (Fig. 6b).

Although phosphoregulation of Bcl-XS might well rely on several signalling cascades, we focused on the Bcl-XS(S106A) mutant as it best

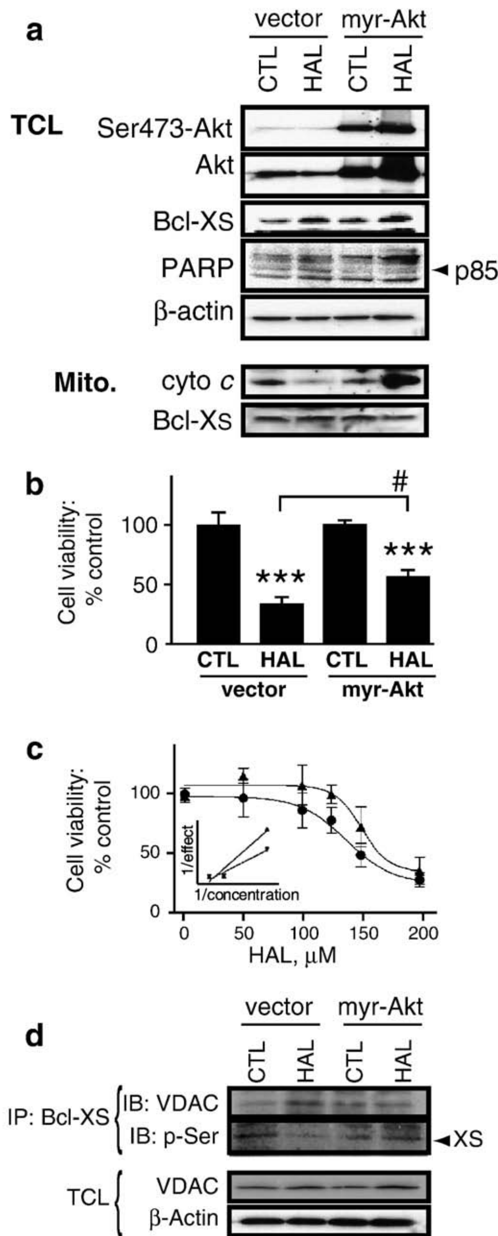

Fig. 4. Membrane-directed, constitutively active Akt diminishes the effects of HAL. (a) PC12 cells were transfected with the CS2+ vector (vector) or with the constitutively active Akt (myr-Akt) expression plasmid. Precleared protein extracts were analyzed by Western blot for Akt phosphorylation, Bcl-XS expression and PARP cleavage to the p85 fragment. The mitochondrial fraction was examined for cytochrome *c* (cyto *c*) and Bcl-XS expression. (b) PC12 cell viability (based on MTT conversion) was determined in cultures expressing myr-Akt (24 h) and treated with vehicle control (CTL) or HAL (125 μM, 24 h). (c) HAL dose–response curves (0–200 μM, 24 h) were generated in PC12 cells overexpressing the plasmid vector (●) or myr-Akt (■) ($n=6$). (c, *insert*). Data from (c) is represented as a double-reciprocal plot. (d) PC12 cells were transfected/treated as in (a) above and protein extracts (precleared by non-specific IgG) were immunoprecipitated for Bcl-XS. The resolved proteins were analyzed by Western blot with either a pan-phospho-Serine (p-Ser) antibody (the molecular weight of the p-Ser signal is approximately 26 kDa and corresponds to that of Bcl-XS) and with anti-VDAC. Total cell lysates (TCL) were probed for VDAC and β-actin (included as a loading control).

suited the testing of our hypothesis of an Akt-dependent regulation of Bcl-XS function. Bcl-XS(S106A) toxicity in PC12 cells coincides with cytosolic translocation of cytochrome *c* and the cleavage of PARP (Fig. 6c). In contrast, Bcl-XS(S106A) toxicity in HEK293A cells coincides with a loss of VDAC expression, the retention of cytochrome *c* in the mitochondria and the absence of PARP cleavage (Fig. 6c).

3.7. The mutation of Bcl-XS on Serine106 promotes its association with VDAC and hinders its association with Akt

FLAG-Bcl-XS and FLAG-Bcl-XS(S106A) were expressed in PC12 cells (Fig. 7) and corresponding protein extracts were immunoprecipitated for FLAG. VDAC is detected in Bcl-XS(S106A), but not wildtype Bcl-XS, immunocomplexes (Fig. 7a). In contrast, Akt is detected in wildtype FLAG-Bcl-XS, but not in Bcl-XS(S106A), immunocomplexes (Fig. 7b), while Bcl-XS wildtype, but not Bcl-SX(S106A), is detected in Akt immunoprecipitates (Fig. 7b). These data provide strong support that the S106 residue in Bcl-XS is a target for Akt.

3.8. Bcl-XS(S106A) is toxic to cortical neurons

Given that the toxicity associated with the Bcl-XS(S106A) mutant supports a Bcl-XS and an Akt-dependent component to HAL-induced apoptosis in PC12 cells, we chose to examine the potential for cytotoxicity of the Bcl-XS(S106A) mutant in primary cortical cultures. Overexpression of Bcl-XS(S106A), confirmed by anti-FLAG Western blot analysis, results in an increase in cleavage of caspase-3 to the active p17 fragment (Fig. 8, top panel) and a corresponding increase in chromatin condensation in primary neuronal cultures compared to vector-transfected controls (Fig. 8, bottom panel).

4. Discussion

Changes in Akt function have been linked to antipsychotics, including HAL, and schizophrenia [21,22,24]. The concentration of

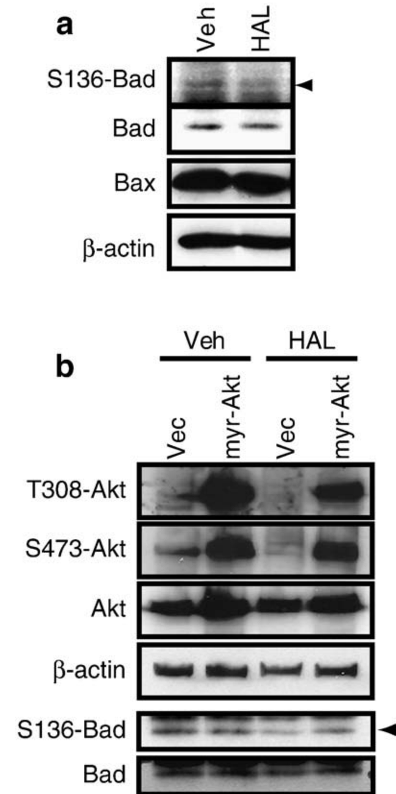

Fig. 5. Activated Akt rescues the HAL-induced dephosphorylation of Bad-Ser136 in PC12 cells. (a) PC12 cells treated with HAL (125 μM, 24 h) and cell lysates were analyzed by Western blot for the phosphorylation status of Bad and for the expression of Bax (two pro-apoptotic Bcl-2-related proteins). (b) PC12 cells expressing the activated myr-Akt (24 h) were treated with HAL (125 μM, 24 h). Cell lysates were then examined to confirm Akt phosphorylation on Thr308 (T308-Akt) and Ser473 (S473-Akt), and to examine the effect of Akt on Bad Serine136 (S136-Bad) phosphorylation. β-Actin demonstrates equal protein loading.

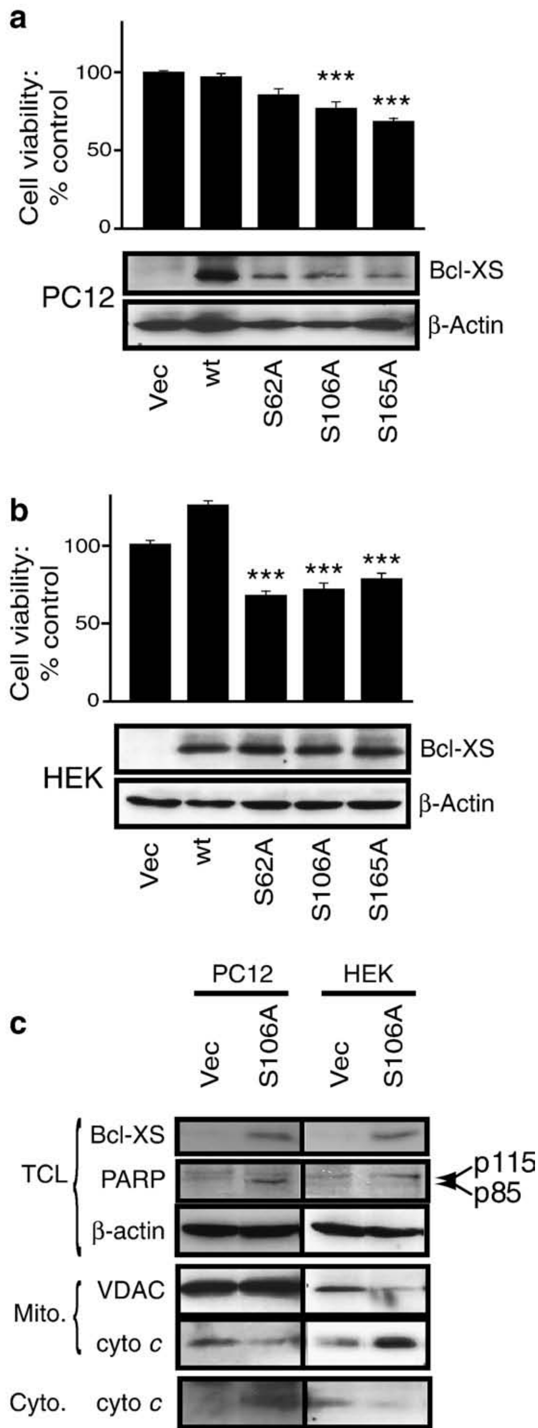

Fig. 6. Bcl-XS species lacking the potential for phosphorylation at selected serine residues exert toxicity in a cell line-dependent manner. MTT conversion was used as a means of examining the cytotoxicity of overexpressed Bcl-XS proteins bearing Ser-to-Ala substitutions, *e.g.* Ser62, Ser106 and Ser165, in (a) PC12 cell and (b) HEK293A cell cultures. (c) Bcl-XS(S106A) was overexpressed in PC12 and HEK293A cells. The protein extracts were either used to confirm Bcl-XS(S106A) expression or PARP cleavage. Similar extracts were used to examine mitochondrial VADC and/or cytoplasmic cytochrome *c* (cyto *c*) expression.

lipophilic drugs, such as HAL, in the brain in both laboratory and clinical settings are 20–100 times higher than that in corresponding plasma (*e.g.* it can reach 10 μM in brain; discussed in ref. [33]). Therefore, the μM concentrations of HAL used herein are reasonable and similar to those used in other *in vitro* studies of HAL [19,20,28,34,35].

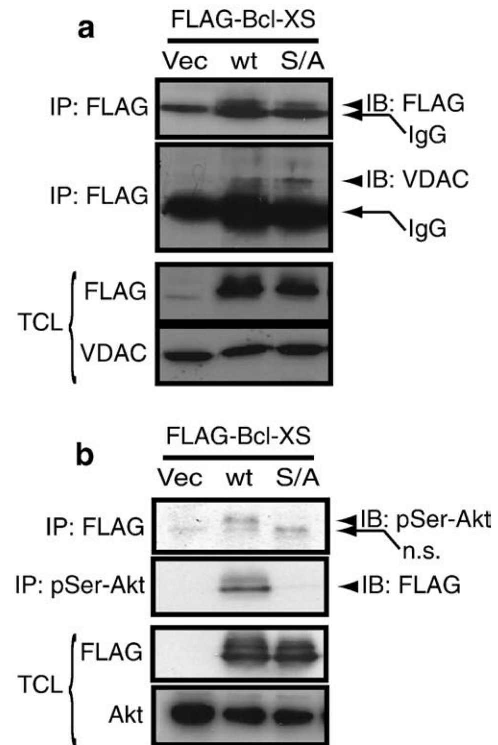

Fig. 7. Bcl-XS(S106A) associates differently with VDAC and Akt than does wildtype Bcl-XS. FLAG-tagged Bcl-XS wildtype (wt) or S106A (S/A) was expressed in HEK293A cell cultures. (a) IgG-precleared FLAG immunoprecipitates (IP: FLAG) were examined for VDAC (IB: VDAC). FLAG-Bcl-XS (wt or S106A) overexpression and VDAC expression were confirmed in corresponding total cell lysates (TCL). (b) In separate lysates, FLAG immunoprecipitates were examined for activated Akt (Ser473; pSer-Akt) and pSer-Akt immunoprecipitates (IP: pSer-Akt) were examined for FLAG (IB: FLAG). IgG: the light chain of the immunoprecipitating antibody; n.s.: a non-specific band detected at 50 kDa, slightly below the 55 kDa Akt-specific band.

We have previously observed that HAL treatment of PC12 cells actually results in induction of p85-associated PI3K lipid kinase activity [21], which was initially counterintuitive given that Akt was

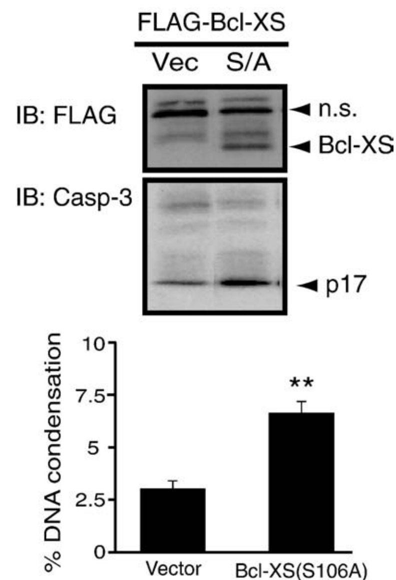

Fig. 8. Bcl-XS(S106A) is toxic to primary neuronal cultures. FLAG-Bcl-XS(S106A) was expressed in primary cortical neurons for 24 h and protein extracts were examined for Bcl-XS(S106A) (S/A) expression (using anti-FLAG) and caspase-3 cleavage to the active p17 fragment. Chromatin condensation (visualized using Hoechst nuclear staining) was used to determine the number of apoptotic nuclei (t=4.931, df=4; P=0.0079).

concurrently inactivated. However, we also demonstrated that the active PI3K complex translocated to the nucleus, but that phosphoinositide-dependent protein kinase 1 (PDK1), the direct upstream regulator of Akt, remained in the cytoplasm of these cells; this differential compartmentalization of PI3K and PDK1 effectively uncoupled PI3K and PDK1, resulting in the disruption of Akt function [21]. Our current pharmacological demonstration of non-competitive antagonism between HAL and Akt confirms that HAL inactivates the PI3K/Akt pathway *via* a mechanism that is not targeting Akt directly.

The Ser106 residue in Bcl-XS is a new target for Akt and its dephosphorylation, *i.e.* the S106A substitution, promotes a cytochrome *c*-dependent apoptotic phenotype in PC12 cell. This clearly reflects the ability for Akt to phosphoregulate Bcl-2 homologues' homo- and heterodimerization [36] as well as their subcellular localizations and their roles in apoptosis [2,37,38]. Yet, while Bcl-XS (S106A) cytotoxicity also relies on a mitochondrial event in HEK293A cells, it does not rely on the release of cytochrome *c*. It does, however, reduce the expression of the VDAC, which is an integral component of the mitochondrial pore complex that channels not only cytochrome *c*, but ATP as well. Thus, the loss of VDAC and the disruption of the pore complex would effectively disrupt ATP flux and any ATP-dependent mechanisms, ultimately affecting cell function [39]. This is supported indirectly by the conspicuous absence in these cells of [caspase-dependent] PARP cleavage (an ATP-sensitive process in HEK cells; ref. [40]). Fridman and colleagues [41,42], also using non-neuronal cultures had already observed that the mitochondria of Bcl-XS-expressing 3T3 fibroblasts were less electron-dense or even transparent, indicating lack of membrane content, and that this coincided with the cytosolic depletion of cytochrome *c*, with the absence of any chromatin condensation and with caspase-independent death.

Chronic HAL treatment, apparently *via* the antagonism of the dopamine D2 receptor, exerts positive effects on Akt function *in vivo* [23,43]. In contrast, Akt inactivation following *acute* HAL treatment of PC12 and primary neuronal cultures is independent of the dopamine D2 receptor. HAL is already known to occasionally function independently of the D2 receptor, as evidenced, for example, with c-*fos* induction in ventral tegmental area dopamine neurons in primary culture [44].

σ1/σ2 receptors have affinity for psychotropic drugs, including HAL, which is known to function as a σ1 receptor antagonist and as a σ2 receptor agonist. If HAL were acting simply as a σ1 receptor antagonist, then it would clearly mitigate this receptor's "neuroprotective" role (reviewed in [45]) in both PC12 cells and N2-a neuroblastoma cells, both of which express the σ1 receptor at comparable levels [20]. Yet, HAL is more cytotoxic to neuroblastoma N2a cells ($LC50 = 80 \pm 5$ μM) than it is to PC12 cells ($LC50 = 147 \pm 12$ μM), thus precluding an effect specific to antagonism of the σ1 receptor [20]. In this study HAL is also more cytotoxic to cortical cultures ($LC50 = 57 \pm 16$ μM) than it is to PC12 cultures ($LC50 = 138 \pm 5$ μM). σ2 receptors are localized to lipid rafts and intracellular compartments [46,47] and their activation requires compounds, such as HAL, that are sufficiently lipophilic so as to penetrate or completely cross any membrane. These combined data support the notion that HAL-induced apoptosis in these cells is more likely a result of σ2 receptor activation. The contribution of the σ2 receptor system to HAL-induced inactivation of Akt function (and to the resulting dephosphorylation of both Bcl-XS and Bad) certainly needs to be explored further.

The fact that HAL elicits caspase-dependent and -independent events in neuronal and non-neuronal cell lines reflects what is already known of σ2 receptor agonist-induced cell death. Indeed, the σ2 receptor mediates mitochondrial depolarization in both SK-N-SH neuroblastoma cells and MCF-7/Adr breast tumour cells; yet in SK-N-SH cells the cell death is caspase-dependent and okadaic acid-insensitive, whereas in the MCF7 cells it is caspase-independent and okadaic acid-sensitive [48,49]. This effect is apparently Bax-dependent in the breast tumour cells, but not in the neuroblastoma cells, which corroborates our own conclusions of HAL mediating a Bax-independent mechanism in the neuronal PC12 cell line ([20]; present study). This also confirms Bcl-XS-mediated apoptosis as being distinct from Bax-dependent apoptosis in PC12 cells [25] and in embryonic fibroblasts [31]. In the latter cells, Bcl-XS might interfere with a Bcl-XL/Bak heterodimer, thereby releasing pro-apoptotic Bak from the complex [31] in much the same way that Bad can interfere with a Bcl-XL/Bax complex to release Bax for ultimate mitochondrial targeting [50].

We have previously shown that apoptosis consistently accounts for approximately 25% of HAL-induced PC12 cytotoxicity [20]. During the present study, treatment with the PI3K inhibitor LY294002 resulted in approximately 20% apoptotic cells (as determined using chromatin condensation) and this is not significantly different from that resulting from HAL treatment alone or with the combination of HAL and LY294002; this suggests that both compounds are targeting the same pathway and that the apoptotic component of HAL cytotoxicity is dependent on inactivation of the PI3K/Akt pathway. The fact that constitutively active Akt can only reverse a portion of HAL-induced cytotoxicity further supports this notion. Obviously, additional signalling cascades might also be involved; in fact, JNK (*e.g.* given that it targets Ser62 in Bcl-XL: [12]) or ERK1/2 (given its role in the multisite phosphoregulation of Bad: refs. [15,30]) both could theoretically contribute to Bcl-XS-Ser62 phosphorylation and needs further examination. The toxicity associated with the C-terminal Bcl-XS-Ser165 residue is reminiscent of the toxicity associated with the dephosphorylation of the homologous site in Bax, *i.e.* Ser184, which is located in the C-terminal tail that targets Bax for insertion into the mitochondrial membrane [2]. The flanking regions of Bcl-XS-Ser165 and Bax-Ser184 do not remotely resemble the canonical RxRxxS/T Akt consensus motif [1], yet Akt has been clearly linked to Bax-Ser184 phosphorylation [2] and, thus, could exert similar influence on the function of Bcl-XS-Ser165. Both non-canonical Akt-dependent phosphorylation of Ser127 in the Yes-associated protein [51] and canonical Akt-dependent phosphorylation of Ser136 in Bad [52] enhance their binding with the cytoplasmic sequestering protein, 14-3-3. Although HAL regulates Akt-mediated phosphorylation of Bad and Bcl-XS (arguing for similarities in their regulation), Bcl-XS is not detectable in 14-3-3 immunocomplexes (or *vice versa*) (YD, JQ and DDM; unpublished data). Thus, sequestration by 14-3-3 does not appear to be the primary means for cytoplasmic retention of Bcl-XS in naïve cells.

Inhibition of caspase-3 also affords partial protection, again arguing that multiple effector pathways might contribute to HAL/σ2 receptor-induced apoptosis, even in the same cell. In support of this notion, σ ligands (*e.g.* CB-64D and HAL) can induce concurrent caspase-8- and -10-dependent cleavage of Bid, and the release of AIF, endonuclease-G, and cytochrome *c* in SK-N-SH cells [53].

While these data provide for a molecular effect of HAL, what is their implication for the use of HAL in the clinical setting? HAL is still used as a primary therapeutic for psychosis in many countries and is also often used "off-label" (*i.e.* as a tranquilizer because of its "calming effects", rather than as an antipsychotic) to treat agitation, often in elderly or demented patients and/or to treat delirium in the post-operative setting. Unfortunately, behavioral and cognitive dysfunction are both exacerbated by antipsychotics such as HAL in both healthy elderly volunteers and Alzheimer disease patients [54–56], which is perhaps not surprising given that σ ligands and receptors have been associated with several neuropsychiatric disorders as well as in age-related changes in higher-ordered brain functions such as memory and learning [57]. We have recently demonstrated a cytotoxic synergism between HAL and the Alzheimer disease-related peptide β-amyloid [20], while ongoing investigations (Z. Wei, G.G. Gabriel, D. Zhang and D.D. Mousseau) indicate that a single injection of HAL to the TgCRND8 mouse (an aggressive model of amyloidosis and Alzheimer disease) elicits sustained cognitive deficits (over several

days) that reflect changes in Bcl-XS expression and its association with VDAC. The role of Akt in this context, however, remains unclear.

In conclusion, there are cell line-dependent effects of HAL on Akt/Bcl-XS phosphorylation that clearly affect mitochondrial function and that undoubtedly contribute to the toxic profile of HAL. While this would clearly hinder its use as an antipsychotic or for its use "off label" as a tranquilizer, this same mechanism could subserve the use of HAL and other σ2 receptor agonists in situations where apoptosis is desirable, such as *acute* therapeutics in drug-resistant cancers (which often have elevated levels of σ2 receptor expression) and for pathological angiogenesis [58–65].

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