A VITAL ROLE FOR Kv CHANNELS IN DOPAMINE TRANSPORTER-MEDIATED 6-HYDROXYDOPAMINE NEUROTOXICITY

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Abstract

6-hydroxydopamine (6-OHDA), a neurotoxic substrate of the dopamine transporter (DAT), is widely used in Parkinson's disease models. However, the molecular mechanisms underlying 6-OHDA's selectivity for dopamine neurons and the injurious sequelae that it triggers are not well understood. We tested whether ectopic expression of DAT induces sensitivity to 6-OHDA in non-dopaminergic cortical neurons and evaluated the contribution of Kv channel-dependent apoptosis to the toxicity of this compound in cortical and midbrain dopamine neurons. Cortical neurons expressing DAT accumulated dopamine and were highly vulnerable to 6-OHDA. Pharmacological inhibition of DAT completely blocked this toxicity. We also observed a p38-dependent Kv current surge in DAT-expressing cortical neurons exposed to 6-OHDA, and p38 antagonists and Kv channel blockers were neuroprotective in this model. Thus, DAT-mediated uptake of 6-OHDA recruited the oxidant-induced Kv channel dependent cell death pathway present in cortical neurons. Finally, we report that 6-OHDA also increased Kv currents in cultured midbrain dopamine neurons and this toxicity was blocked with Kv channel antagonists. We conclude that native DAT expression accounts for the dopamine neuron specific toxicity of 6-OHDA. Following uptake, 6-OHDA triggers the oxidant-associated Kv channel-dependent cell death pathway that is conserved in non-dopaminergic cortical neurons and midbrain dopamine neurons.

Keywords

potassium channels; dopamine transporter; 6-hydroxydopamine; neurotoxicity; p38; mitogen activated protein kinase

Parkinson's disease is characterized by the loss of midbrain dopaminergic neurons. For over 35 years, the catecholamine-derived neurotoxin 6-hydroxydopamine (6-OHDA) has been used to induce dopaminergic cell death in various models of this disorder (Przedborski and Ischiropoulos, 2005). However, at sufficiently high concentrations all catecholamine derivatives, including dopamine and norepinephrine, induce non-selective cell death due to the

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generation of reactive oxygen species and reactive quinones following auto-oxidation (Heikkila and Cohen, 1972; Rosenberg, 1988; Kumar et al., 1995; Hanrott et al., 2006). As such, the selectivity of 6-OHDA for dopaminergic neurons, under the right experimental circumstances, may be due to the distinct neurochemical properties of this cell type and/or the fact that the catecholaminergic toxin acts as a substrate for the dopamine transporter (DAT), unique to these cells (Blum et al., 2001). Nonetheless, in spite the widespread use of 6-OHDA, the precise molecular mechanism by which this toxin destroys dopaminergic neurons has not been fully delineated, although many mechanisms have been proposed (Blum et al., 2001). Here, we first examined whether DAT expression in non-dopaminergic neurons would be sufficient to render these cells selectively sensitive to 6-OHDA toxicity. In addition, the molecular mechanism of 6-OHDA-induced cell death in this artificial system was later confirmed to exist in dopaminergic neurons, revealing a potentially new neuroprotective strategy for Parkinson's disease and related disorders.

EXPERIMENTAL PROCEDURES

Green enhanced fluorescent protein, GFP, was from Clontech (Palo Alto, CA). GFP-DAT cDNA was kindly provided by Dr. S. Amara (Pittsburgh, PA, USA). Luciferase, pUHC13-3, was from Dr. H. Bujard (Heidelberg, Germany). Tyrosine hydroxylase antibody was from Chemicon (Temecula, CA, USA). Stromatoxin was purchased from Alomone (Jerusalem, Israel). Freshly prepared 6-OHDA (Sigma Chemical Co., St. Louis, MO, USA) stocks (10 mM) were prepared in 0.15% ascorbate and kept at $-80°C$ until ready to use. Exposure vehicle consisted of minimum essential medium (without phenol red) with 25 mM HEPES and 0.01% bovine serum albumin. Solutions of 100 μM 6-OHDA in the exposure vehicle were prepared immediately (<1 min) prior to use, as they auto-oxidized at neutral pH at a rate of approximately 30% per minute at room temperature, as measured spectrophotometrically (490 nm). No further auto-oxidation products were noted beyond 6–7 minutes. Cortical cultures were prepared from E16 rat embryos as previously described (Hartnett et al., 1997). Cultures were transfected at 18–22 DIV with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA; Pal et al., 2003; ~5% transfection efficiency). Cells were maintained for 24 to 48 hours at 37°C, 5% CO_2 prior to recording and toxicity assays. [^{3}H]-Dopamine (DA) uptake assays were performed as described in Prasad and Amara (2001). Uptake was performed for 5 minutes at room temperature in Ringer's solution containing 1 μM catechol-O-methyltransferase inhibitor Ro 41-0960 (Sigma), and 50 nM ^{3}H-dopamine (60 Ci/mmol; PerkinElmer, Wellesley, MA, USA). Ventral mesencephalic cultures were established from postnatal day 1 rat (Cardozo and Bean, 1995; Hahn et al., 2006). Briefly, dissected tissue was dissociated with 20 U/ml papain (Worthington Biochemicals, Lakewood, NJ, USA) and triturated. The cell suspension was layered onto Basal Medium Eagle solution containing 10 mg/ml trypsin inhibitor 10 mg/ml bovine serum albumin and spun for 8 min at 200 g. The pellet was resuspended and cells plated at a density of 35,000 cells per 31 mm poly-L-lysine-coated glass coverslip. Cultures were fed twice weekly with Basal Medium Eagle with N2 supplement (Gibco, Carlsbad, CA, USA), penicillin/ streptomycin, 2% rat serum, 0.6 mM glutamine, 10 mM glucose, and 10 mM HEPES; pH 7.3. Electrophysiological and toxicity experiments were performed at >12 DIV. At this *in vitro* age, functional expression of the DAT is well established (Valchar and Hanbauer, 1995) and, in our hands, the cells become highly vulnerable to 6-OHDA. We compared the sensitivity of dopaminergic neurons to 6-OHDA toxicity (100 μM, 15 min exposure) at two developmental stages, 8 DIV (n=5) and 15 DIV (n=9). We observed that viability of the younger cells was much grater than in the older cells following the toxin exposure ($84.7 \pm 15.4\%$ at 8 DIV vs. $44.2 \pm 5\%$ at 15 DIV; $p<0.01$).

Recordings were conducted using the whole-cell configuration of the patch-clamp technique as described previously (McLaughlin et al., 2001). The extracellular solution contained (in mM): 115 NaCl, 2.5 KCl, 2.0 MgCl2, 10 HEPES, 10 D-glucose, pH 7.2, 0.1 mM tetrodotoxin.

The electrode solution contained (in mM): 120 K-gluconate, 11 EGTA, 10 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, 0.22 ATP; pH 7.2 and osmolarity adjusted to 280 mOsm with sucrose. Measurements were obtained under voltage clamp with an Axopatch 200 amplifier (Axon Instruments, Foster City, CA) and pClamp software (Axon Instruments) using 2 MΩ electrodes. Partial compensation (80%) for series resistance was always performed. Currents were filtered at 2 kHz and digitized at 10 kHz (Digidata; Axon Instruments). Potassium currents were evoked with a series of incremental positive voltage steps from a negative holding potential. Steady-state current amplitudes were leak-subtracted (P/N protocol), and normalized to cell capacitance. Capacitive transients were subtracted manually with the amplifier built-in circuits. Transfected cortical neurons were identified by GFP fluorescence. DA neurons were identified by fluorescence by 5,7-dihydroxytryptamine (Silva et al., 1988; Cardozo and Bean, 1995; Hahn et al., 2003). We found that 5,7-dihydroxytryptamine incubation could diminish the neurotoxic consequences of 6-OHDA exposure. As such, 6-OHDA treatments always preceded labeling.

Toxicity assays in cortical neurons were conducted at 48 hours post-transfection in parallel with electrophysiological recordings, but in luciferase co-transfected cells (Boeckman and Aizenman, 1996; Pal et al., 2003), or, in untransfected cells by a lactate dehydrogenase release assay (Hartnett et al., 1997). Cells were rinsed immediately prior to drug treatment. Cells were exposed to either vehicle or 6-OHDA for 15 min at 37°C, 5% CO_2. Luciferase activity as an index of cell viability (Boeckman and Aizenman, 1996) was measured using the GeneLux Kit (Perkin Elmer, Boston, MA, USA) in a Victor2 Multilabel Counter (Perkin Elmer). Since we found that the potassium channel blocker tetraethylammonium (TEA) interfered with luciferase expression, toxicity assays involving this drug were quantified by cell counting of GFP-positive neurons. Counts were obtained from 30 fields of a 40X objective per condition, in triplicate. Toxicity in mesencephalic cultures (15 DIV) was determined by cell counts of tyrosine hydroxylase positive cells 24 hours after 6-OHDA treatments. Counts were conducted by determining the total number of TH positive cells in the entire culture dish, with at least 2–3 culture dishes per condition, using a 20X objective.

RESULTS

We first provide evidence of a functional separation between dopaminergic neuronal phenotype and DAT function in the toxicity of 6-OHDA. Non-dopaminergic rat cortical neurons in culture were transfected with a plasmid encoding a GFP-DAT fusion protein (Fig. 1A), and its functional expression confirmed by [^{3}H]-dopamine uptake. We observed a significant increase in [^{3}H]-DA uptake in GFP-DAT expressing cells, when compared to neurons transfected with a GFP-only expression vector (Fig. 1B). We then tested whether the catecholaminergic neurotoxin 6-OHDA would preferentially injure the GFP-DAT-expressing cortical neurons. A 15 min exposure to 100 μM 6-OHDA, which was relatively innocuous to untransfected cortical neurons (Fig. 1C), or cortical neurons expressing GFP-only vector (Fig. 1D), was sufficient to kill approximately 50% of the GFP-DAT expressing cortical neurons (Fig. 1D). In contrast, the glutamatergic toxin N-methyl-D-aspartate (200 μM, 15 min) killed neurons equally well, regardless of the expression of GFP-DAT (Fig. 1D). The toxicity of 6-OHDA in GFP-DAT-expressing cortical neurons was dependent on the activity of the transporter as it was completely blocked by the DAT inhibitor GBR12909 (Fig. 1E). This situation is highly reminiscent of the conditions necessary to induce 50% apoptotic cell death in dopaminergic neurons in vitro (Ding et al., 2004). As such, the expression of DAT is necessary and sufficient to render non-dopaminergic neurons selectively susceptible to 6-OHDA toxicity under conditions where the extracellular auto-oxidation of the toxin induces little or no non-specific injury.

Dopaminergic neurons treated with 6-OHDA undergo caspase-dependent cell death following the phosphorylation of the mitogen-activated protein kinase (MAPK) p38 (Choi et al., 2004). As such, we tested whether the p38 antagonist SB239063 could block 6-OHDA-induced toxicity in GFP-DAT expressing cortical neurons. We observed that SB239063 was indeed very effective in preventing cell death induced by the neurotoxin (Fig. 1E). We have previously reported that oxidative stress in cortical neurons leads to a p38-dependent enhancement of voltage-dependent K^+ currents (McLaughlin et al., 2001). This K^+ current surge, mediated by the membrane insertion of Kv2.1-encoded K^+ channels, is required for apoptosis to occur, and precedes caspase activation (McLaughlin et al., 2001; Pal et al., 2003; Pal et al., 2006). Therefore, we investigated if 6-OHDA activated a similar, p38-dependent pathway in GFP-DAT expressing cortical neurons. A pronounced, toxin-induced K^+ current surge was observed in these cells, which was completely suppressed by SB239063 (Fig. 2A). More critically, the K^+ channel blocker tetraethylammonium (TEA; Fig. 2B) was neuroprotective against 6-OHDA in GFP-DAT expressing cortical neurons, similar to what we have observed for other oxidant stressors (McLaughlin et al., 2001). These data show that DAT-dependent 6-OHDA toxicity can activate a cell death pathway that is very similar to that triggered by other oxidants in cortical neurons.

Given these results, we investigated whether 6-OHDA could induce a K^+ current surge in dopaminergic neurons, which express DAT endogenously. In addition we tested whether K^+ channel antagonists could prevent 6-OHDA toxicity in these cells. Similar to what was observed in GFP-DAT expressing cortical neurons, 6-OHDA induced approximately a 2-fold increase in voltage-dependent K^+ currents in dopaminergic neurons obtained from rat mesencephalic cultures (Figs. 3A and B). In addition, this toxin (100 μM, 15 min) produced, approximately, 50% dopaminergic neuronal cell death (Fig. 3C). Importantly, 6-OHDA toxicity to dopaminergic neurons could be completely abrogated by 10 mM TEA (Fig. 3C). We also observed nearly complete neuroprotection by the Kv2.1-selective blocker stromatoxin (ScTX, 100 nM; Shiau et al., 2003;Grishin et al., 2005;Fig. 3C), but not by the K-ATP channel antagonist glibenclamide (1 μM; not shown). These results indicate that 6-OHDA toxicity in dopaminergic cells activates a K^+ current surge, reminiscent to what has been reported many cell types undergoing apoptosis (Yu, 2003;Bortner and Cidlowski, 2004). Additionally, blocking K^+ channels was neuroprotective, suggesting their critical role in the cell death process.

DISCUSSION

The selectivity of 6-OHDA for catecholaminergic neurons, and, especially, dopaminergic neurons, has been the subject of much debate due to the fact that this substance, and indeed all catecholaminergic-derived compounds, can auto-oxidize in physiological solutions to generate highly reactive quinones, as well as free radicals (Heikkila and Cohen, 1972; Rosenberg, 1988; Kumar et al., 1995; Hanrott et al., 2006). Nonetheless, as early as 1970, it was suggested that 6-OHDA could selectively damage dopaminergic neurons *in vivo* due to the long-term depletion of central dopamine following intracerebral injections of the toxin (Uretski and Iversen, 1970). The sparing of 6-OHDA-treated cultured dopaminergic neurons by DAT inhibitors provided unequivocal evidence that, at least under certain conditions, the selectivity of the toxin could be accounted by cellular uptake systems (Cerruti et al., 1993). The results presented in this study corroborate this finding and indicate that under conditions where extracellular auto oxidation of 6-OHDA is not sufficient to induce non-selective injury (Hanrott et al., 2006), the presence of DAT activity can render the catecholaminergic derivative toxic, even in neurons not having an otherwise dopaminergic phenotype. As such, DAT expression is not only sufficient, but a decisive component for the manifestation of selective 6-OHDA toxicity.

Cellular K^+ efflux is a requisite step for apoptosis to proceed in a variety cell death paradigms (Yu, 2003;Bortner and Cidlowski, 2004). The enhancement of potassium currents during the apoptotic process leads to a decrease in the concentration of this cation in the cytoplasm, which acts as a permissive cell death signal (Yu, 2003;Bortner and Cidlowski, 2004). Our group has demonstrated that the K^+ efflux accompanying oxidant-induced apoptosis is mediated by an enhancement of voltage-gated K^+ currents (McLaughlin et al., 2001). This phenomenon is triggered by the oxidative liberation of intraneuronal Zn^{2+}, which leads to a p38-mediated exocytotic insertion of Kv2.1-encoded channels (Aizenman et al., 2000;McLaughlin et al., 2001;Pal et al., 2003;Pal et al., 2006). Recently, however, it was demonstrated that activation of ATP-sensitive potassium channels (K-ATP) was critical for the selective vulnerability of nigral dopaminergic neurons to the mitochondrial complex I inhibitors rotenone and 1-methyl-4-phenylpyridinium (Liss et al., 2005). However, the molecular link between K-ATP channel activation and cell death pathways was not established, nor was it demonstrated whether these channels were also associated with 6-OHDA-induced toxicity. The lack of neuroprotection by the K-ATP channel blocker glibenclamide observed in our studies indicates that these channels are not directly involved in the neurotoxicity induced by 6-OHDA in mesencephalic cultures. Nonetheless, it is entirely plausible that different K^+ channels are able to provide various exit routes for the cation, even in the same cell type, depending on the injurious stimulus. While metabolically-sensitive K-ATP channels may be ideally suited to detect alterations in mitochondrial function by complex 1 inhibitors (Liss et al., 2005), voltage-gated channels like Kv2.1, which are directly linked to oxidative signaling processes (Pal et al., 2004), mediate 6-OHDA toxicity, and possibly the toxicity of other oxidants. It is noteworthy that the general term "oxidative stress" is commonly and intimately associated with nigral cell death in the Parkinson's disease literature (Abou-Sleiman et al., 2006). The neuroprotective actions of ScTX, a Kv2.1-selective blocker, observed here, suggest that this channel mediates K^+ efflux in dopaminergic neurons undergoing apoptosis, just like it does in cortical cells (Pal et al., 2003). Interestingly, Kv2.1 has recently been shown to be a major binding partner of DAT (Maiya et al., 2006). Our data strongly indicates that K^+ channels may provide novel therapeutic targets for neuroprotection in Parkinson's disease and related disorders.

Acknowledgements

Abbreviations

DA

dopamine

DAT

dopamine transporter

DIV

days *in* vitro

GFP

green fluorescent protein

6-OHDA

6-hydroxydopmaine

Kv

voltage-dependent potassium channel

ScTX

stromatoxin

TEA

tetraethylammonium

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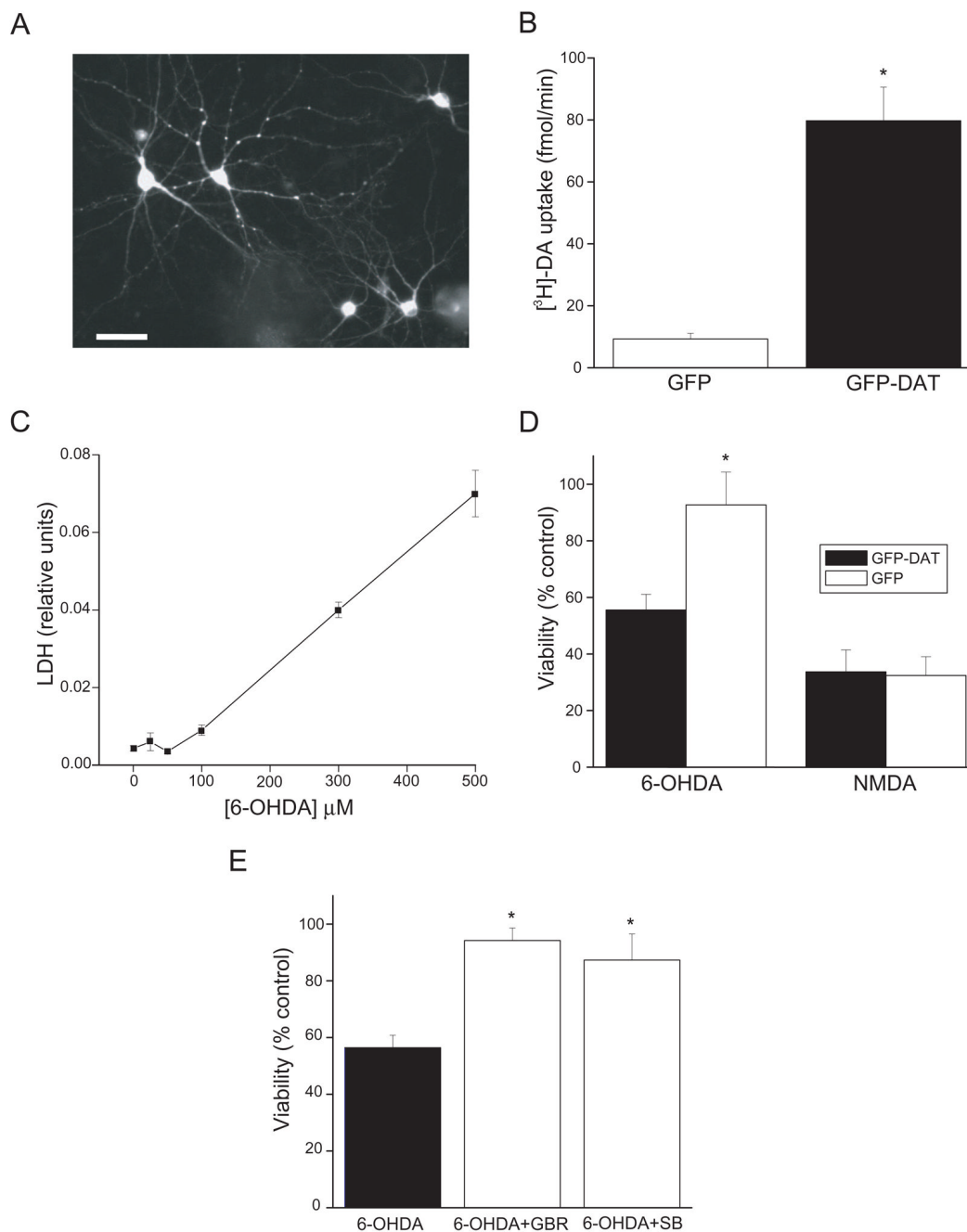**Figure 1.**
DAT renders non-dopaminergic neurons sensitive to 6-OHDA. *A,* Micrograph showing the expression of GFP-DAT in transfected cortical neurons (bar, 80 μm). ***B***, Expression of GFP-DAT in cultured cortical neurons leads to functional expression of the transporter as measured by [^{3}H]dopamine (DA) uptake, which is not observed in GFP vector alone-expressing cultures. Values represent the m ± s.e.m. uptake (fmol/min) in three independent experiments (*p < 0.005; t-test). Inset: GFP-DAT expressing neuron. ***C***, Concentration-toxicity relationship for naïve, untransfected cortical neurons exposed to increasing levels of 6-OHDA for 15 min. Toxicity was measured by a lactate dehydrogenase (LDH) release assay (n=4). ***D***, 6-OHDA is toxic to GFP-DAT expressing cortical neurons. GFP-DAT and GFP expressing cortical

neurons were exposed to 6-OHDA (100 μM, 15 min) or to NMDA (200 μM, 15 min). Viability was assayed by a luciferase cell survival assay 24 hr. later and expressed as a percent of vehicle-treated control (n=6–10). Note that 6OHDA induced 50% cell death in GFP-DAT expressing neurons but was not lethal to GFP-expressing cells. In contrast, NMDA was equally toxic to both types of neurons (*p<0.001; t-test). *E*, 6-OHDA toxicity in GFP-DAT expressing cortical neurons is blocked by a DAT uptake inhibitor and by a p38-MAPK antagonist. GFP-DAT expressing cortical neurons were exposed 100 μM 6OHDA (15 min) in the absence or presence of either 10 μM GBR12909 (a DA uptake inhibitor) or 20 μM SB29063 (a p38 inhibitor). Viability was assayed by a luciferase cell survival assay. Note that both these drugs prevented 6OHDA toxicity (n= 4–13; *p<0.01; ANOVA/Dunnet).
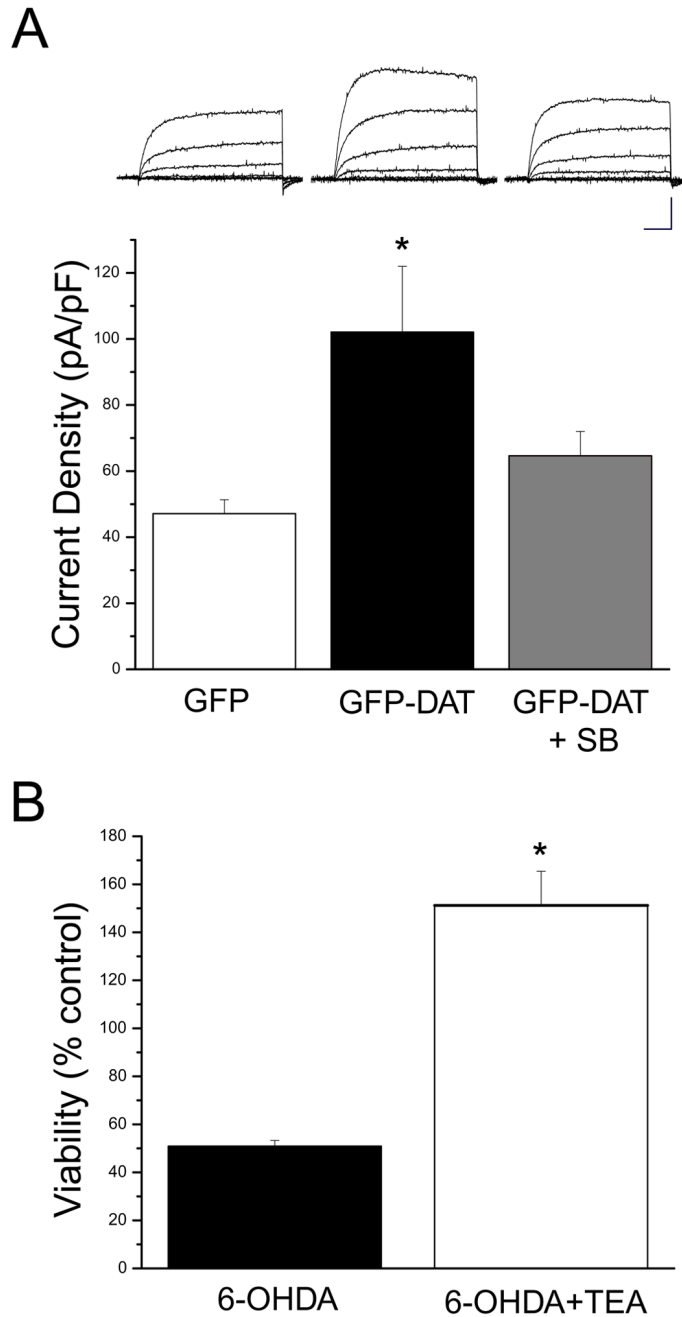
Figure 2.
Critical role of Kv channels in 6-OHDA toxicity in GFP-DAT expressing cortical neurons.
A, 6-OHDA enhances voltage-dependent K$^+$ currents in GFP-DAT expressing cortical neurons. Bottom, mean current densities in wild type cortical neurons (left; n = 21), GFP-DAT expressing cortical neurons (middle; n = 16) and GFP-DAT expressing cortical neurons pretreated with the p38 inhibitor SB293063 (20 μM, right; n = 10). All cells were treated with 30 μM 6-OHDA for 15 min prior to recording (100 μM of the toxin led to very unstable recordings in GFP-DAT expressing neurons). Currents were evoked by a voltage-step to +5 mV from a holding potential of −70 mV and normalized to cell capacitance (*p<0.05; ANOVA/ Dunnet). Top, representative whole-cell recordings from all three treatment groups. Currents

were evoked by a series of 15 mV voltage steps to +35 mV from −70 mV. Each set of traces is directly above its corresponding treatment group displayed in the bar graph. Calibration: 5nA, 15ms Note that the 6-OHDA-induced current surge could be blocked by the p38 inhibitor. **B**, 6-OHDA toxicity in GFP-DAT-expressing cortical neurons can be blocked by a K^+ channel inhibitor. GFP-DAT expressing cortical neurons were exposed 100 μM 6OHDA (15 min) in the absence or presence of either 10 mM TEA. TEA treatment continued following toxin exposure. Viability was assayed by cell counting (GFP-positive cells) 24 hr. later as TEA interfered with the luciferase expression assay utilized above. Note TEA protected cells from 6OHDA toxicity (n=3; *p<0.005, t-test).
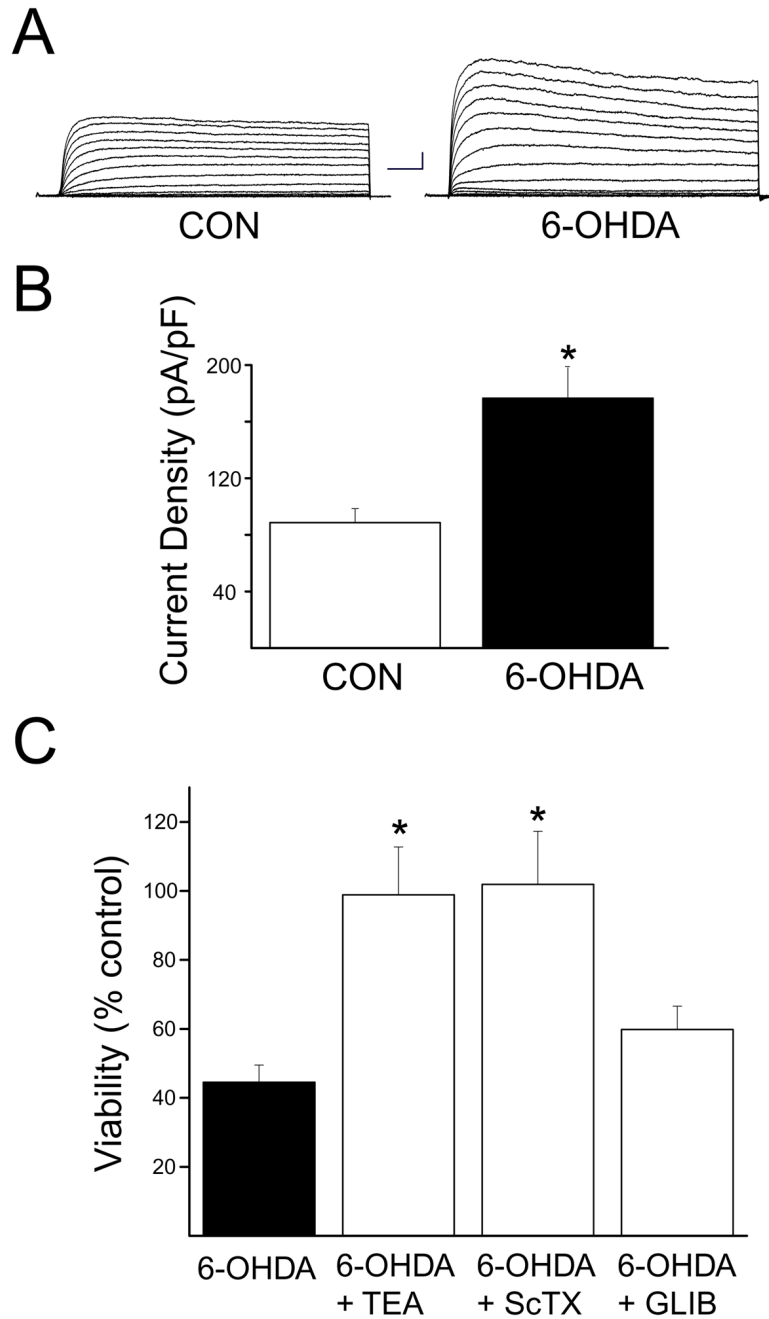
Figure 3.
Kv channels mediate 6-OHDA toxicity in dopaminergic neurons. *A,* 6-OHDA enhances voltage-dependent K^+ currents in mesencephalic dopaminergic neurons. Currents were evoked from 13 DIV dopaminergic cells in mesencephalic cultures by a series of 10 mV voltage steps to +80 mV from a holding voltage of −80 mV under control conditions and following a 15 min treatment with 30 μM 6-OHDA. *B*, Mean ± s.e.m. current densities under control (n =10) and 6-OHDA treatment (n = 6) conditions. Currents were evoked by a voltage step to +10 mV from a holding potential of −80 mV and normalized to cell capacitance (*p<0.001; t-test). (**g**) 6-OHDA toxicity in dopaminergic neurons can be blocked by TEA and stromatoxin, but not glibenclamide. Mesencephalic cultures (15 DIV) were treated with 100 μM 6OHDA (15 min)

in the absence or presence of 10 mM TEA, 100 nM stromatoxin (ScTX), or 1 μM glibenclamide (GLIB). TEA, ScTX and GLB treatments continued following toxin exposure. Cultures were fixed and immunostained for the presence of tyrosine hydroxylase (TH) 24 hours later. TH positive cells were then counted to assay DA cell survival. Note TEA and STR, but not GLIB, protected cells from 6-OHDA toxicity (n=3–9; *p<0.01; ANOVA/Dunnet). TEA, STR, and GLIB alone did not affect their viability (not shown).