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Inhibition of L-Type Ca^{2+} Channels by TRPC1-STIM1 Complex Is Essential for the Protection of Dopaminergic Neurons

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Loss of dopaminergic (DA) neurons leads to Parkinson's disease; however, the mechanism(s) for the vulnerability of DA neurons is(are) not fully understood. We demonstrate that TRPC1 regulates the L-type Ca^{2+} channel that contributes to the rhythmic activity of adult DA neurons in the substantia nigra region. Store depletion that activates TRPC1, via STIM1, inhibits the frequency and amplitude of the rhythmic activity in DA neurons of wild-type, but not in $TRPC1^{-/-}$, mice. Similarly, $TRPC1^{-/-}$ substantia nigra neurons showed increased L-type Ca^{2+} currents, decreased stimulation-dependent STIM1-$Ca_v1.3$ interaction, and decreased DA neurons. L-type Ca^{2+} currents and the open channel probability of $Ca_v1.3$ channels were also reduced upon TRPC1 activation, whereas increased $Ca_v1.3$ currents were observed upon STIM1 or TRPC1 silencing. Increased interaction between $Ca_v1.3$-TRPC1-STIM1 was observed upon store depletion and the loss of either TRPC1 or STIM1 led to DA cell death, which was prevented by inhibiting L-type Ca^{2+} channels. Neurotoxins that mimic Parkinson's disease increased $Ca_v1.3$ function, decreased TRPC1 expression, inhibited Tg-mediated STIM1-$Ca_v1.3$ interaction, and induced caspase activation. Importantly, restoration of TRPC1 expression not only inhibited $Ca_v1.3$ function but increased cell survival. Together, we provide evidence that TRPC1 suppresses $Ca_v1.3$ activity by providing an STIM1-based scaffold, which is essential for DA neuron survival.

Key words: calcium; Cav1.3; Parkinson's disease; SOCE; TRPC1-STIM1

Significance Statement

Ca^{2+} entry serves critical cellular functions in virtually every cell type, and appropriate regulation of Ca^{2+} in neurons is essential for proper function. In Parkinson's disease, DA neurons are specifically degenerated, but the mechanism is not known. Unlike other neurons, DA neurons depend on $Ca_v1.3$ channels for their rhythmic activity. Our studies show that, in normal conditions, the pacemaking activity in DA neurons is inhibited by the TRPC1-STIM1 complex. Neurotoxins that mimic Parkinson's disease target TRPC1 expression, which leads to an abnormal increase in $Ca_v1.3$ activity, thereby causing degeneration of DA neurons. These findings link TRPC1 to $Ca_v1.3$ regulation and provide important indications about how disrupting Ca^{2+} balance could have a direct implication in the treatment of Parkinson's patients.

Introduction

Parkinson's disease (PD) is a progressive, hypokinetic, neurodegenerative disease. Although the cause of PD is not well understood, it is known that the loss of dopaminergic (DA) neurons in the substantia nigra (SN) region underlies the main motor symptoms for this disease but the mechanism is still not clear. In recent

years, attention has turned to the role of calcium (Ca^{2+}) in the onset of PD. Ca^{2+}-induced excitotoxicity has been considered an important mechanism leading to DA neuron cell death. However, not all of the DA midbrain neurons are equally affected by the degenerative process in PD. In particular, SN DA neurons are highly vulnerable to being affected via environmental factors and toxins that lead to PD compared with more resistant neighboring VTA DA neurons (Björklund and Dunnett, 2007; Dragicevic et al., 2015). It has been proposed that sodium channels play a major role in the spike generation of VTA DA cells, whereas Ca^{2+} channels could contribute to the pacemaking activity in SN DA neurons.

L-type Ca^{2+} channels, mainly Ca$_v$1.3, have been shown to contribute to the rhythmic activity of DA neurons in the SN region and could render DA neurons susceptible to mitochondrial toxins (Guzman et al., 2010; Surmeier et al., 2011; Dryanovski et al., 2013). Importantly, an increase in Ca$_v$1.3 expression was observed in PD brains, which precedes PD pathology (Hurley et al., 2015). In addition, a change in the ratio of Ca$_V$1.2 versus Ca$_V$1.3 was observed that could contribute to Ca^{2+}-mediated excitotoxicity (Guzman et al., 2010; Kang et al., 2012; Hurley et al., 2015). Consistent with these studies, patients treated with Ca$_v$1 antagonists had a decreased risk for PD (Ritz et al., 2010; Marras et al., 2012). However, in PD, not all DA neurons degenerate and most neurons start to degenerate at the later stage of life, suggesting that there could be alternative mechanisms that may inhibit Ca$_v$1.3 activity and help in the survival of DA neurons.

Changes in Ca^{2+} homeostasis with regard to storage organelles, such as the ER and mitochondria, have also been shown to affect neuronal survival and are closely linked with PD (Kazuno et al., 2006). Ca^{2+} entry through the store-operated Ca^{2+} entry (SOCE) channels is essential for maintaining intracellular Ca^{2+} stores thus could regulate neuronal function and survival (Bollimuntha et al., 2006; Selvaraj et al., 2012). TRPC1 has been suggested as a candidate for SOCE-mediated Ca^{2+} entry and has been shown to be critical for neuronal survival (Wang and Poo, 2005; Bollimuntha et al., 2006; Selvaraj et al., 2012). In addition, stromal interacting molecule-1 (STIM1) has been identified as the primary regulator of SOCE-mediated Ca^{2+} entry, and store depletion allows STIM1 rearrangement followed by its interaction with TRPC1 that induces Ca^{2+} entry (Huang et al., 2006; Pani et al., 2012). Interestingly, STIM1 has also been shown to inhibit L-type Ca^{2+} channels (Park et al., 2010; Wang et al., 2010); however, the mechanism as to how STIM1 inhibits L-type Ca^{2+} channels is not known. Furthermore, a role for SOCE-mediated Ca^{2+} entry in the regulation of Ca$_v$1.3 channels in DA neurons has yet to be established. Thus, the objective of this study was to identify the relationship between TRPC1 and Ca$_v$1.3 channels and establish their role in regulating DA neuronal function. Our previous studies have shown that TRPC1 channels are important for DA neuronal function and protect against cytotoxicity induced by neurotoxins by inhibiting ER stress (Selvaraj et al., 2009, 2012). Results presented here indicate, for the first time, that TRPC1 is essential for the inhibition of L-type Ca^{2+} channels. DA neurons in TRPC1$^{-/-}$ mice showed increased spontaneous rhythmic activity as well as increased degeneration of DA neurons. Furthermore, the frequency of neuronal firing was increased in DA neurons of TRPC1$^{-/-}$ mice and TRPC1-inhibited L-type Ca^{2+} channel, especially Ca$_v$1.3 activity, by facilitating an STIM1-Ca$_v$1.3 interaction, which was essential for the protection of DA neurons.

Materials and Methods

Cell culture, transfections, and viability assays. SH-SY5Y cells were obtained from the ATCC and cultured/maintained at 37°C with 95% humidified air and 5% CO$_2$ and differentiated as previously described (Bollimuntha et al., 2005; Selvaraj et al., 2012). For transient transfection, SH-SY5Y cells were transfected with TRPC1siRNA (Ambion) or Cav1.3 siRNA or STIM1siRNA or scrambled control siRNA (Ambion negative control siRNA #1) using HiPerfect transfection reagent (QIAGEN). For rescue experiments, cells were transfected with siSTIM1 for 24 h followed by overexpression of full-length STIM1 (10-fold concentration). For viability assays, cells were seeded in 96-well plates at a density of 0.5×10^5 cells/well. The cultures were grown for 24 h under different conditions, and cell viability was measured by using the MTT method as described previously (Selvaraj et al., 2012).

Animals and human brain samples. Two- to 5-week-old male C57BL/6 (Charles River) or TRPC1$^{-/-}$ mice were used for these experiments. The TRPC1$^{-/-}$ mice were generated as previously described (Liu et al., 2007). All animals were housed in a temperature-controlled room under a 12/12 h light/dark cycle with *ad libitum* access to food and water, and experiments were performed as per the institutional guidelines for the use and care of animals. Frozen and paraffin-embedded blocks of postmortem human SN samples of control (6 samples, males with age ranging from 65 to 72 years) and PD patients (8 samples, males and females with age ranging from 60 to 72 years, with severe motor neuron deficit) were obtained from United Kingdom Parkinson's foundation as well as Udall Parkinson Center (Philadelphia). All human subjects were approved by the institutional review board. Immunofluorescence was performed on sections using specific antibodies as described.

Brain slice preparation and electrophysiology. Mice (2–5 weeks old) were killed, and the brain was dissected in ice-cold saline solution. Coronal brain sections (400 μm) were cut using a vibrating blade microtome (VT1000S; Leica). Brain slice recordings were performed under infrared videomicroscopy (Olympus BX51WI) as described previously (Zhang et al., 2014). Recording electrodes were filled with (in mM) as follows: 100 K$^+$-gluconate, 0.6 EGTA, 2 MgCl$_2$, 8 NaCl, 33 HEPES, 2 ATPNa$_2$, 0.4 GTPNa, and 7 phosphocreatine, pH 7.4. The extracellular solution comprised the following (in mM): 130 NaCl, 24 NaHCO$_3$, 3.5 KCl, 1.25 NaH$_2$PO$_4$, 2.5 CaCl$_2$, 1.5 MgCl$_2$, and 10 glucose, saturated with 95% O$_2$ and 5% CO$_2$, pH 7.4. For calcium current recordings, TTX is added in the extracellular solution and K$^+$-gluconate is replaced with CsCL in intracellular solution. For SOCE current recordings, recording electrodes were filled with the following (in mM): 110 CsCL, 5 MgCl$_2$, 10 EGTA, 10 HEPES, 4 ATPNa$_2$, and 0.1 GTPNa, pH 7.4. Treatments applied after the firing had been stable for 5–10 min. Amplitude and frequency were calculated by Mini Analysis 6.0.1 (Synaptosoft). For Ca^{2+} currents, cells were voltage-clamped using a train of three action potentials (APs) that showed spontaneous firing and was used to establish the time course of Ca^{2+} currents under AP trains (Marcantoni et al., 2010; Vandael et al., 2012). For single-cell recordings, coverslips with SH-SY5Y cells were transferred to the recording chamber and perfused with an external Ringer's solution of the following composition (mM): 125 TEA-Cl, 10 CaCl$_2$, 10 HEPES, 10 glucose, pH 7.4 (Tris). The patch pipette was filled with the standard intracellular solution that contained the following (mM): 130 CsCL, 2 MgCl$_2$, 20 HEPES, 11 EGTA, 2 Na$_2$ATP, 0.1 GTP, 10 glucose, pH 7.3 (CsOH). All electrophysiological experiments were filtered at 2 kHz, digitized at 10 kHz, and acquired and analyzed using pCLAMP 10 software (Molecular Devices). All experiments were performed at room temperature.

Single-channel recording. For single-channel recording in cell-attached configuration, recording electrodes were filled with the following (in mM): 100 TEA-Cl, 10 BaCl$_2$, 10 HEPES, pH 7.4 (Tris). The extracellular solution used to set the membrane potential to zero comprised of the following (in mM): 104 K-aspartate, 1 MgCl$_2$, 10 HEPES, 10 EGTA, pH 7.4. Data were obtained in response to membrane depolarizing pulse, 200 ms duration, from an intracellular holding −40 mV, filtered at 2 kHz, digitized at 10 kHz, and acquired and analyzed using pCLAMP10 software (Molecular Devices). A threshold level was set at one-half of the estimated unitary current amplitude. The low-variance analysis was used

to estimate single-channel current levels. Briefly, the mean current amplitude and variance were calculated for a window advanced across the record in 1 point increments. If the variance in the window was below the baseline variance, the mean current amplitude was kept for the histogram. The histogram bin width was 0.05 pA for all experiments. All experiments were performed at room temperature.

Calcium measurement. Cells were incubated with 2 μM fura-2 (Invitrogen) for 45 min and washed twice with Ca^{2+} free standard external solution, including the following: 10 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1 mM MgCl$_2$, 10 mM glucose, pH 7.4, buffer. For fluorescence measurements, the fluorescence intensity of fura-2-loaded control cells was monitored with a CCD camera-based imaging system (Compix). A monochromator dual-wavelength enabled alternative excitation at 340 and 380 nm, whereas the emission fluorescence was monitored at 510 nm. The images of multiple cells collected at each excitation wavelength were processed using the C imaging, PCI software (Compix), to provide ratios of fura-2 fluorescence from excitation at 340 nm to that from excitation at 380 nm (F340/F380).

Immunofluorescence and immunohistochemistry. Animals were anesthetized and fixed by perfusion with PBS followed by PFA (4%, w/v) in PBS. The whole brain was removed and fixed again overnight in PFA (3%), cryoprotected in 30% sucrose in PBS for 24 h at 4°C, and then frozen in OCT freezing compound (Ted Pella). Serial cryosections were collected through the entire midbrain (10 μM) and stained using TH-specific antibodies. Secondary antibodies that were conjugated with a fluorophore were used to label individual protein; samples without primary antibody were used as a control. For immunohistochemistry, the Vector ABC Elite kit (Vector Laboratories) was used along with 3'3' diaminobenzoic acid as a chromogen to develop the reaction in the presence of H$_2$O$_2$ (Selvaraj et al., 2009). All samples were examined and images obtained using a Leica and Zeiss Meta confocal microscope. For quantitative measurements, persons blind to the treatment counted the number of TH-positive neurons in the SN pars compacta. Measurements from four to six sections per brain were averaged to obtain one value per subject.

Immunoprecipitation and Western blot analysis. Rabbit polyclonal Ab anti-TRPC1 (catalog #ACC-010), anti-TRPC3 (catalog #ACC-016), anti-Orai1, and anti-Ca$_v$1.3 (catalog #ACC-005) were purchased from Alomone Labs. Goat polyclonal Ab against β-actin (catalog #sc-1616) was obtained from Santa Cruz Biotechnology. Rabbit monoclonal Ab against STIM1 (catalog #4916), cleaved caspase3 (catalog #9664), Bak (catalog #2772), and Bid (catalog #2002) were purchased from Cell Signaling Technology. Cells or SN tissues were stimulated (with and without Tg 2 μM) as indicated in the figure followed by the addition of RIPA buffer to obtain cell lysates from both differentiated SH-SY5Y cells and SN region of the brain. Protein concentrations were determined using the Bradford reagent (Bio-Rad); 25 μg of lysates was resolved on NuPAGE 4%–12% Bis-Tris gels (Invitrogen) and followed by Western blotting as described previously (Bollimuntha et al., 2005; Selvaraj et al., 2009). For immunoprecipitation, 1 mg of total lysate was incubated overnight (4°C) with the primary antibody, followed by the addition of protein A beads for 3 h, washed with high salt buffer, and resolved on the gel.

Intrasubstantia nigral injection. Mice received a unilateral injection of either control virus (Ad-GFP) or Ad-TRPC1 (3×10^7 particles per construct in 3 μl) into the SN. After 1 week of adenovirus injection, mice were challenged with MPTP (MPTP-HCl 25 mg/kg per injection for 5 consecutive days at 24 h intervals). After additional 2 weeks, the brain was removed from the skull and placed dorsal side up. Using a scalpel blade, a coronal cut was made adjacent to the inferior colliculi at bregma ∼−6.36 mm. A second cut was made at bregma ∼−2.54 mm, based on the mouse brain atlas. The ventral midbrain was dissected to ensure that there was no contamination of the hippocampus, cortex, or cerebellum. Brain regions from 2 or 3 animals were pooled for each experiment.

Statistical analysis. Data analysis was performed using Origin 9.0 (OriginLab). Student's paired or unpaired t test or ANOVA was used for statistical analysis as appropriate; N indicates the cells or slices examined.

Differences in the mean values (mean $\pm$ SEM) were considered to be significant at $p < 0.05$.

Results

L-type Ca^{2+} channel contributes to the rhythmic activity of DA neurons in the SN region

The loss of DA neurons in the SN region is a prominent characteristic of PD. Thus, we analyzed the expression of the dopamine transporter (DAT), which is a marker for dopaminergic neurons in control and PD samples. Importantly, expression of DAT protein was decreased in human PD patients (Fig. 1A,B). Furthermore, most of the DAT staining in PD patients coincided with endocytic vesicular staining, suggesting that DA neurons are affected in PD. However, as both DA and GABAergic neurons are present in the SN region that modulates dopamine release and function, electrophysiological experiments were performed in slices obtained from SN region of adults ($p > 28$ d) mice. In the whole-cell configuration, the mean frequency of spontaneous APs in GABAergic neurons was much higher than that observed in DA neurons (Fig. 1C,E). Hyperpolarizing current pulses of 200 pA were also used to distinguish between the two neuronal populations, and APs evoked by current injection showed distinct characteristics between the DA and GABAergic neurons (Matsuda et al., 1987; Yung et al., 1991; Richards et al., 1997; Seutin and Engel, 2010) (Fig. 1D,F). Additionally, consistent with previous reports (Okamoto et al., 2006), DA neurons, exhibited I_h ratio <0.6, whereas GABAergic neurons showed a small I_h (I_h ratio >0.6) (Fig. 1G–I), in accordance with the presence of the two different types of neurons in the SN region.

One of the fundamental questions that need to be addressed is why distinctively DA neurons are vulnerable in PD. Interestingly, most neurons exhibit exclusively TTX (a Na$^+$ channel inhibitor) sensitive autonomous pacemakers; however, in the SN area, DA neurons have shown to be partially dependent on Ca^{2+} for its autonomous pacemaking activity (Surmeier et al., 2011). To study this further, tissue slices from the adult SN area were used and AP activity was recorded. As shown in Figure 2A, B, the addition of TTX completely blocked the rhythmic activity in GABAergic neurons. Similar results were also observed in juvenile (P14-P18 d) DA neurons (Fig. 2C,D), which showed that TTX was also able to block the rhythmic activity in DA neurons. In contrast, the rhythmic activity of aged DA neurons ($p > 28$ d) was only partially decreased by the addition of TTX (Fig. 2E,F). In addition, 20% of cells showed slow oscillatory potentials upon application of TTX (Fig. 2G). Furthermore, the rhythmic activity was facilitated by the application of Bay K8644 (L-type Ca^{2+} channel activator) (Fig. 2H). The addition of nifedipine (an L-type Ca^{2+} channel blocker) along with TTX completely abolished the rhythmic activity of aged DA neurons (Fig. 2I). Importantly, removal of external Ca^{2+} completely inhibited the TTX-resistant rhythmic activity in adult DA neurons (Fig. 2J). Together, these results suggest that the rhythmic activity in aged DA neurons is in part driven by the L-type Ca^{2+} channels. In neurons, L-type Ca^{2+} channels have one of the two α1-subunits: Ca$_v$1.2 or Ca$_v$1.3. The Ca$_v$1.3 subunits are highly expressed in the SN region and open at relatively hyperpolarized potentials (Koschak et al., 2001; Xu and Lipscombe, 2001; Helton et al., 2005; Chan et al., 2007; Surmeier et al., 2011; Hurley et al., 2015). Together, these data suggest that the contribution of L-type Ca^{2+} channels, especially Ca$_v$1.3, could play a key role in DA neuron vulnerability and degeneration.
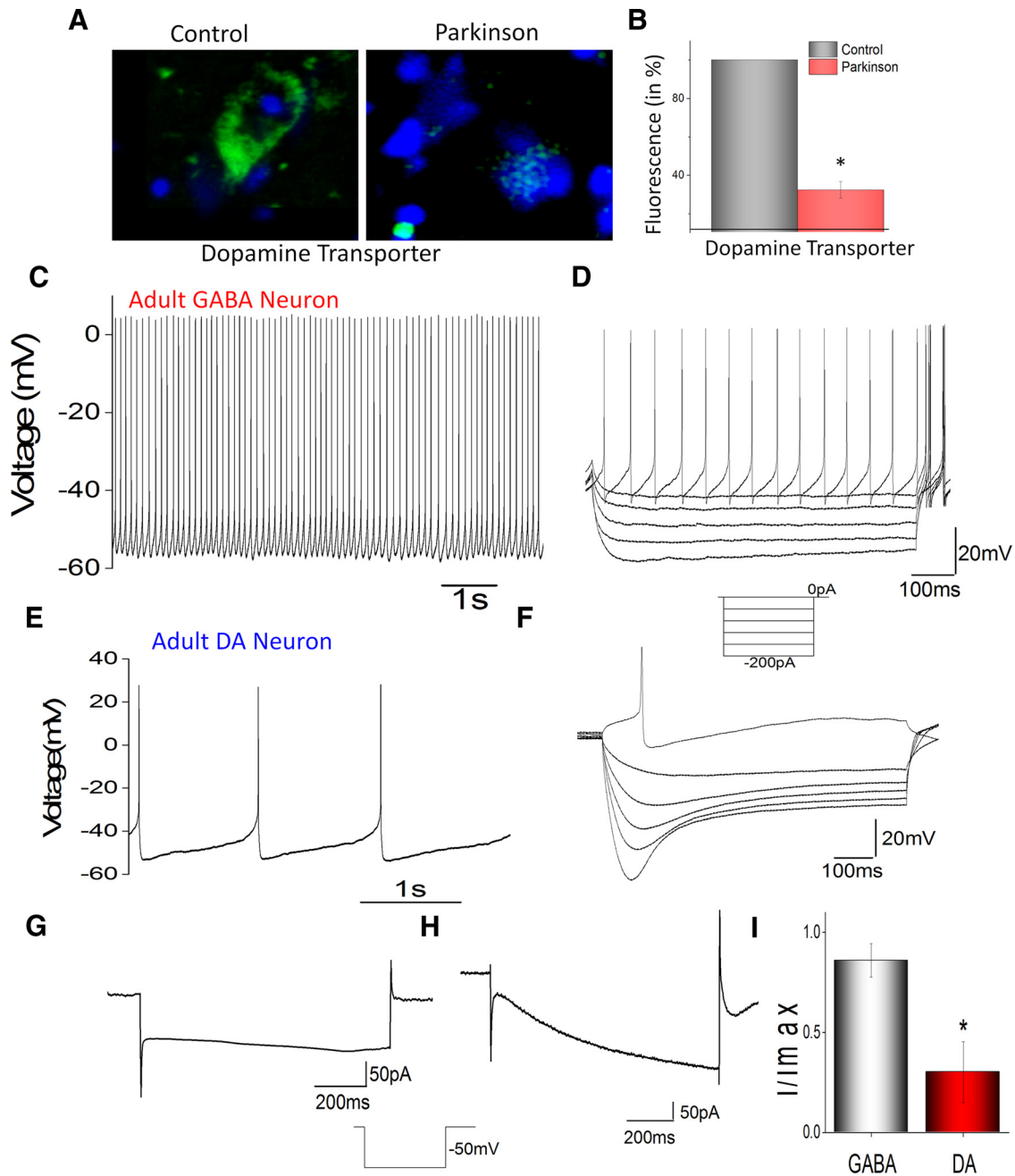
Figure 1. Functional characterization of DA and GABAergic neurons in the SN region. **A**, Paraffin-embedded sections of postmortem human SN region obtained from control and PD patients. Sections were immunostained using DAT. **B**, Quantification of the fluorescence obtained from 5 or 6 individual sections. Patterns of AP firing using tissue slices from SN area in a GABA neuron (**C**) and in adult DA neurons (**E**). Traces were recorded without current injection. **D**, **F**, Voltage traces were evoked by a series of 1000 ms current pulses (from −200 pA with increment −40 pA). **G**, **H**, Current traces evoked by −50 mV steps from holding potential −60 mV. **I**, Bar graph represents I$_h$ ratio in GABA and DA neurons ($n = 15$). *$p < 0.05$.

TRPC1 inhibits the rhythmic activity of DA neurons in the SN area

Our previous studies had shown that another Ca^{2+} channel, TRPC1, is also involved in regulating Ca^{2+} homeostasis and protects against the loss of DA neurons (Bollimuntha et al., 2005; Selvaraj et al., 2012). Thus, given the importance of Ca^{2+} for the rhythmic activity in DA neurons, we further investigated the relationship between L-type Ca^{2+} channels and TRPC1 in aged DA neurons ($p > 28$ d). The spontaneous activity of DA neurons was recorded under the current-clamp mode. Interestingly, tissue slices from SN region of $TRPC1^{−/−}$ mice showed an increase in the AP (1.02 ± 0.07 Hz, $n = 9$) compared with age-matched

wild-type control mice (0.80 ± 0.04 Hz, $n = 9$) (Fig. 3 A, E), suggesting that loss of TRPC1 increases the rhythmic activity of the DA neurons. To further confirm this, TTX was added; and although TTX decreased the rhythmic activity of DA neurons in wild-type mice and $TRPC1^{−/−}$ mice, the rhythmic activity in $TRPC1^{−/−}$ mice (0.41 ± 0.03 Hz, $n = 9$) was slightly higher compared with wild-type DA neurons (0.35 ± 0.04 Hz, $n = 9$) treated with TTX (Fig. 3 B, F, M). The addition of nifedipine, which blocks L-type Ca^{2+} channels, inhibited the pacemaking activity in both wild-type and $TRPC1^{−/−}$ mice (data not shown). More importantly, application of thapsigargin (Tg, a SERCA pump blocker, which activates TRPC1 channels) (Liu et al., 2003;
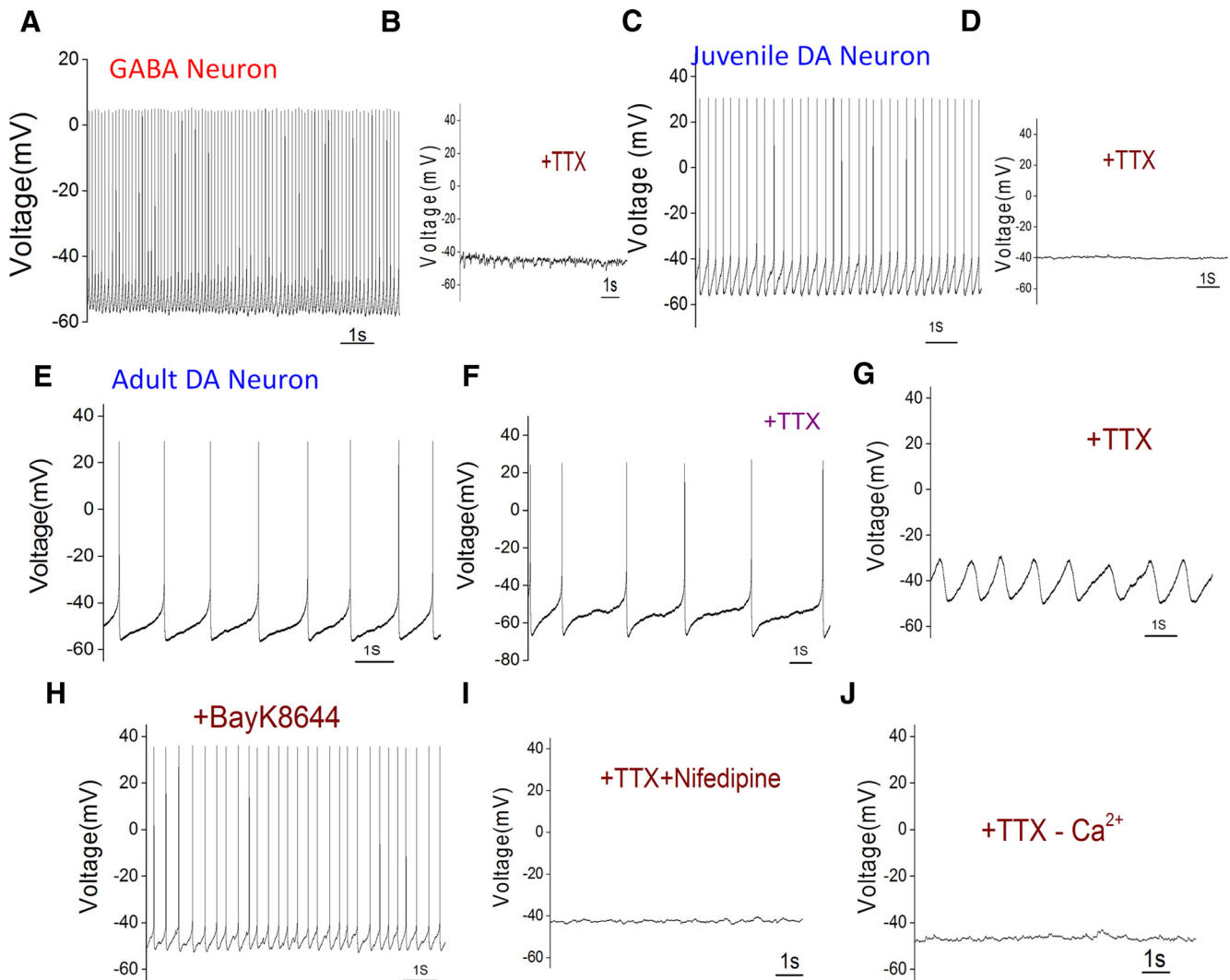
Figure 2. Ca$_v$1.3 channels contribute to the rhythmic activity of DA neurons. In the whole-cell configuration, application of 0.5 μM TTX abolished spiking of GABA neurons in wild-type mice (**A**, **B**). **C**, Spontaneous APs and application of 0.5 μM TTX abolished spiking of DA neurons in juvenile mice (P14 –P18 d) (**D**). **E**, Patterns of AP firing in adult ($p >$ 28 d) DA neurons from the SN area. **F**, **G**, Application of 0.5 μM TTX decreased spiking in adult DA neurons. **H**, Application of 1 μM BayK8644 increased spiking. **I**, The addition of 1 μM nifedipine abolished spiking of DA neurons in adult mice. **J**, Application of 0.5 μM TTX along with removing external Ca^{2+} also abolished spiking of DA neurons in adult mice.

Selvaraj et al., 2012) along with TTX significantly inhibited both the frequency (0.21 $\pm$ 0.05 Hz, $n = 8$) and the amplitude of rhythmic activity of DA neurons in wild-type mice (Fig. 3C, I, N). In contrast, the activity of DA neurons in $TRPC1^{-/-}$ mice was not affected, and no decrease in the frequency (0.38 $\pm$ 0.04 Hz, $n = 8$) or amplitude of the rhythmic activity was observed by the addition of Tg along with TTX (Fig. 3G, J, N). Application of 2-APB (a nonspecific TRPC1 blocker) abolished the effect of Tg-mediated decrease in rhythmic activity in wild-type mice (Fig. 3D, K) (0.35 $\pm$ 0.06 Hz, $n = 6$) but showed no decrease in the rhythmic activity in TRPC1$^{-/-}$ mice (0.40 $\pm$ 0.06 Hz, $n = 6$) (Fig. 3H, L). These results further establish that activation of TRPC1 inhibits spontaneous pacemaking activity in DA neurons.

L-type Ca^{2+} channel activity in the SN region is inhibited by TRPC1
The results shown above suggest that, in DA neurons, TRPC1 inhibits the autonomous activity driven by L-type VGCCs. To confirm that functional TRPC1 channels exist in DA neurons, we stimulated DA neurons with Tg to measure SOCE currents. As indicated in Figure 4A, B, store depletion induced an inward

TRPC1-like current in WT DA neurons, and the current properties were similar as previously reported (Liu et al., 2003; Selvaraj et al., 2012). The addition of 2-APB or knockdown of TRPC1 ($TRPC1^{-/-}$) significantly inhibited SOCE currents that were induced by store depletion (Fig. 4A–C), suggesting that TRPC1 channel functions as a putative SOCE channel in DA neurons. To further confirm the role of TRPC1 in regulating VGCC, we next recorded the Ca^{2+} currents in the tissue slices from the SN area. Importantly, the VGCC activity in $TRPC1^{-/-}$ mice was increased compared with wild-type control mice. Moreover, store depletion, by the addition of Tg, significantly decreased Ca^{2+} currents in wild-type mice, whereas no such decrease in the VGCC activity was observed in $TRPC1^{-/-}$ mice (Fig. 4D–G). The addition of nifedipine was able to block the Ca^{2+} currents in the SN region of wild-type and $TRPC1^{-/-}$ mice (Fig. 4D–F). Similarly, under physiological conditions, VGCC-mediated Ca^{2+} currents were also significantly increased in $TRPC1^{-/-}$ mice compared with wild-type (Fig. 4H, I). Moreover, overload of calcium further increased VGCC-mediated Ca^{2+} currents in $TRPC1^{-/-}$ mice (data not shown). Importantly, no change in the expression of Ca$_v$1.3 or STIM1 or Orai1 was observed in $TRPC1^{-/-}$ mice (Fig.
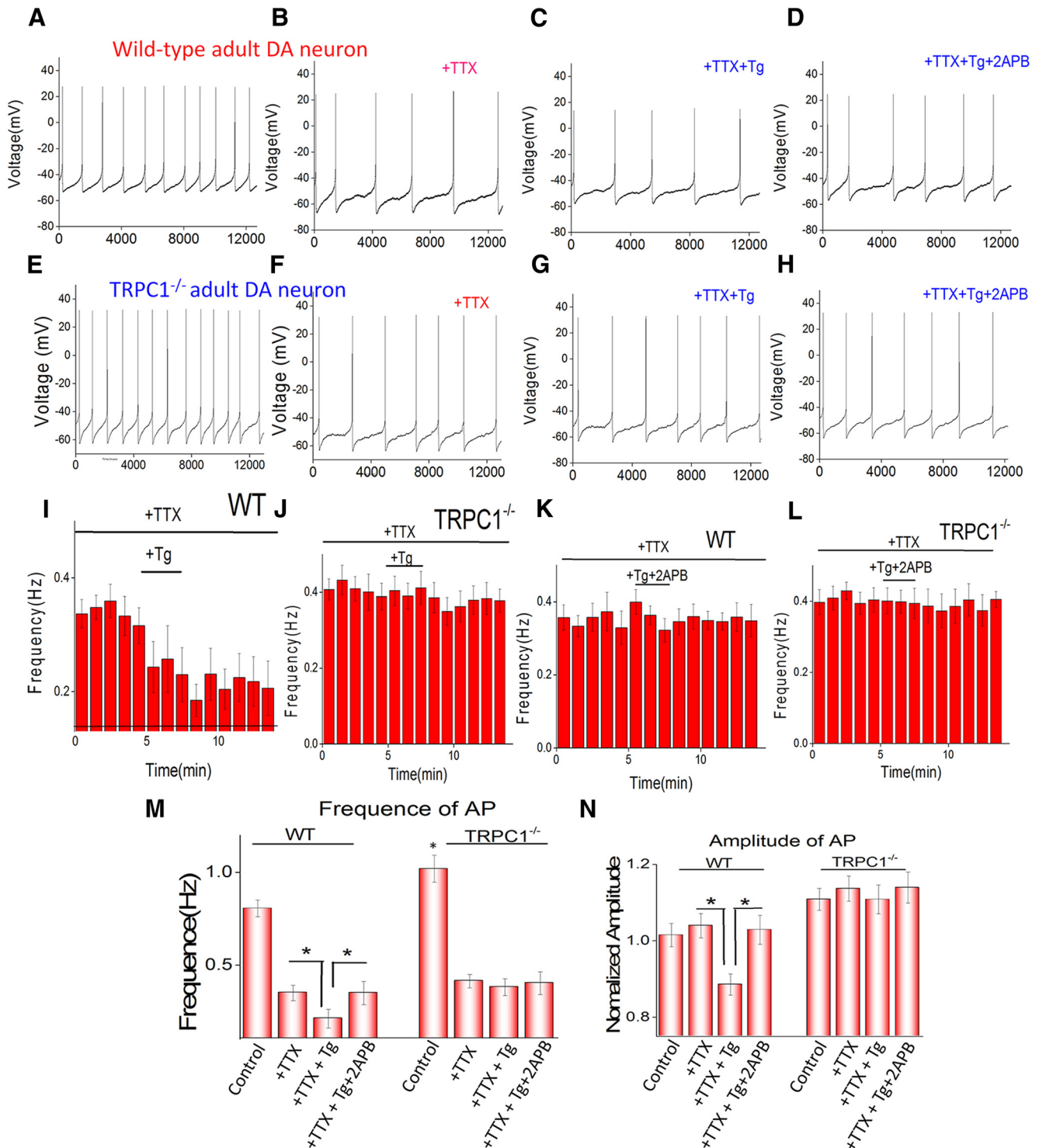
Figure 3. TRPC1 inhibits the rhythmic activity of DA neurons in the SN region. In the whole-cell configuration, patterns of AP firing of adult DA neurons ($p > 28$ d) in wild-type mice (**A**) and *TRPC1*$^{-/-}$ mice (**E**). **B**, **F**, AP firing upon application of 0.5 μM TTX in both wild-type and *TRPC1*$^{-/-}$ slices. **C**, **G**, Rhythmic activity in SN slices that were incubated with 1 μM Tg + TTX in wild-type and *TRPC1*$^{-/-}$ mice, receptively. **D**, **H**, Rhythmic activity in SN slices that were incubated with 1 μM Tg + TTX + 50 μM APB in wild-type and *TRPC1*$^{-/-}$ mice, receptively. **I–L**, Pooled time course of AP firing frequency under various conditions in WT and *TRPC1*$^{-/-}$ mice ($n = 9$). **M**, **N**, The frequencies and normalized amplitude under these conditions. *Significantly different values ($p < 0.05$) ($n = 9$).

4*J*,*K*), suggesting that alterations in Ca^{2+} currents are specifically dependent on TRPC1 expression.

Recent findings have shown that store depletion induces STIM1 aggregation and increases STIM1 interaction with TRPC1, thus activating TRPC1 channels (Huang et al., 2006) that could suppress L-type Ca^{2+} channel currents. These findings led

us to test whether the TRPC1-STIM1 complex interacts with Ca$_v$1.3. Application of 1 μM Tg showed an increase in the pull-down of Ca$_v$1.3 by STIM1 in wild-type mice compared with unstimulated conditions (Fig. 5*A*). However, in *TRPC1*$^{-/-}$ mice, the store-dependent increase in the STIM1-Ca$_v$1.3 association was abolished, suggesting that STIM1 interaction with Ca$_v$1.3 is
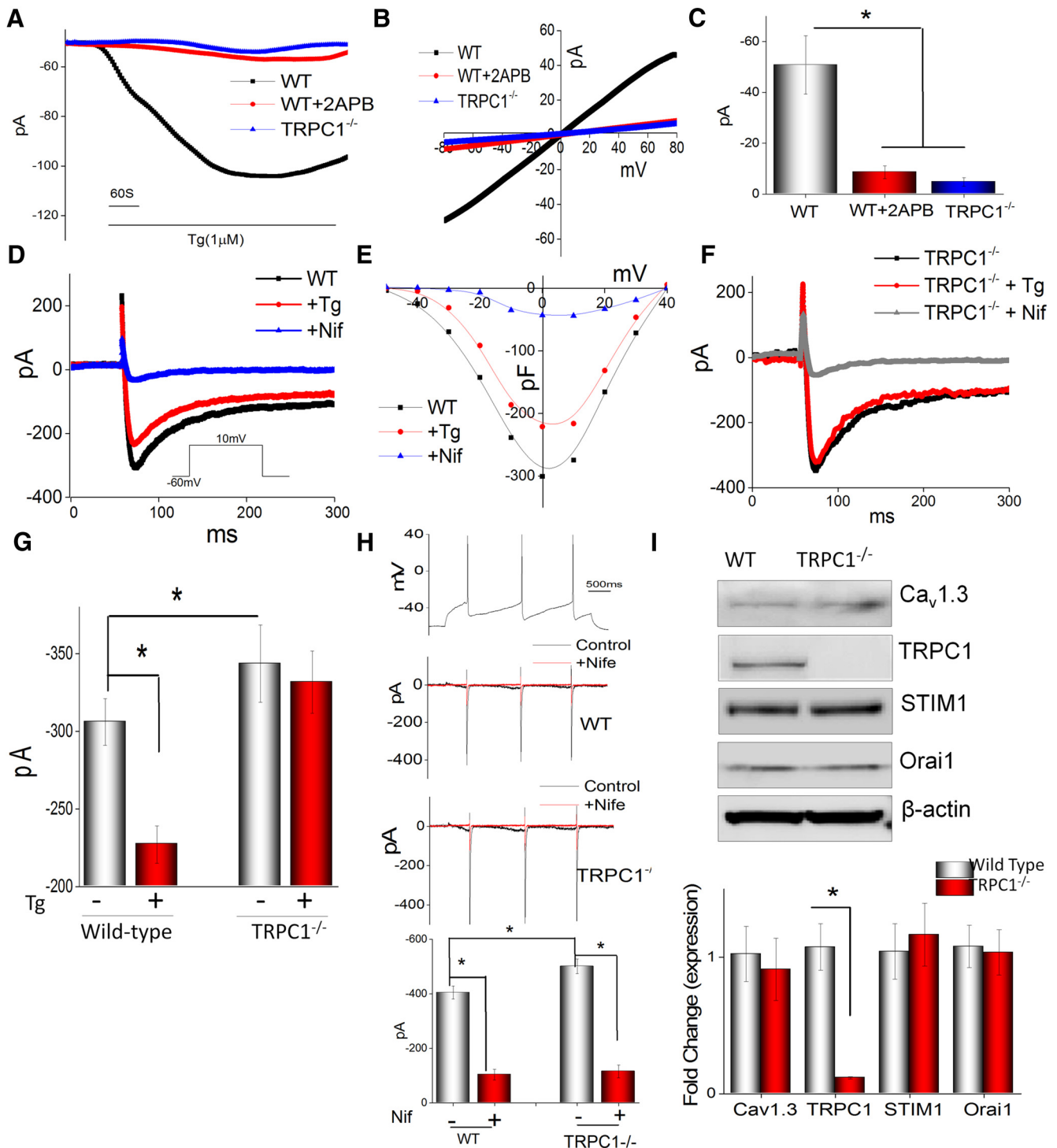
Figure 4. TRPC1 modulates Ca$_v$1.3 activity in SN area of mice. ***A***, Representative trace showing store dependent (upon addition of 1 μM Tg Ca^{2+} entry at −70 mV holding potential) that induced a TRPC1-like current in DA neurons of WT mice but was abolished in the *TRPC1$^{−/−}$* mice. Application 50 μM 2-APB also significantly inhibited the currents in wild-type mice. ***B***, I–V curves under these conditions. ***C***, Maximum current (at −70 mV holding potential) in wild-type with and without 2-APB and in *TRPC1$^{−/−}$* mice (n = 11). ***D***, Representative trace showed that calcium currents evoked in DA neurons from SN area using a step protocol from −60 to 10 mV in the whole-cell configuration in wild-type mice. ***E***, Representative I–V relations under these conditions (n = 8). ***F***, Representative trace showed that Ca^{2+} currents evoked in DA neurons from SN area in *TRPC1$^{−/−}$* mice. ***G***, The average (10–12 recordings) currents under various conditions (n = 10). *p < 0.05. ***H***, A train of three APs induced Ca^{2+} currents in WT and *TRPC1$^{−/−}$* mice (n = 5). *p < 0.05. ***I***, Western blots of lysates from SN tissues from control and *TRPC1$^{−/−}$* mice. Antibodies used are indicated in the figure. Densitometry for normalized Ca$_v$1.3 relative to β-actin bar graph is to show quantitation from at least three independent experiments. Data are mean ± SEM from three independent experiments. *p < 0.05.

dependent on TRPC1 expression. To establish the importance of TRPC1 in DA neurons, we next investigated the presence of DA neurons in the SN area by evaluating the TH expression in wild-type and *TRPC1$^{−/−}$* mice from the mid region (rostral to

caudal). Interestingly, TH-positive neurons showed that DA neurons in the SN region were significantly decreased in *TRPC1$^{−/−}$* mice compared with the wild-type mice. In contrast, DA neurons present in the VTA region showed no such
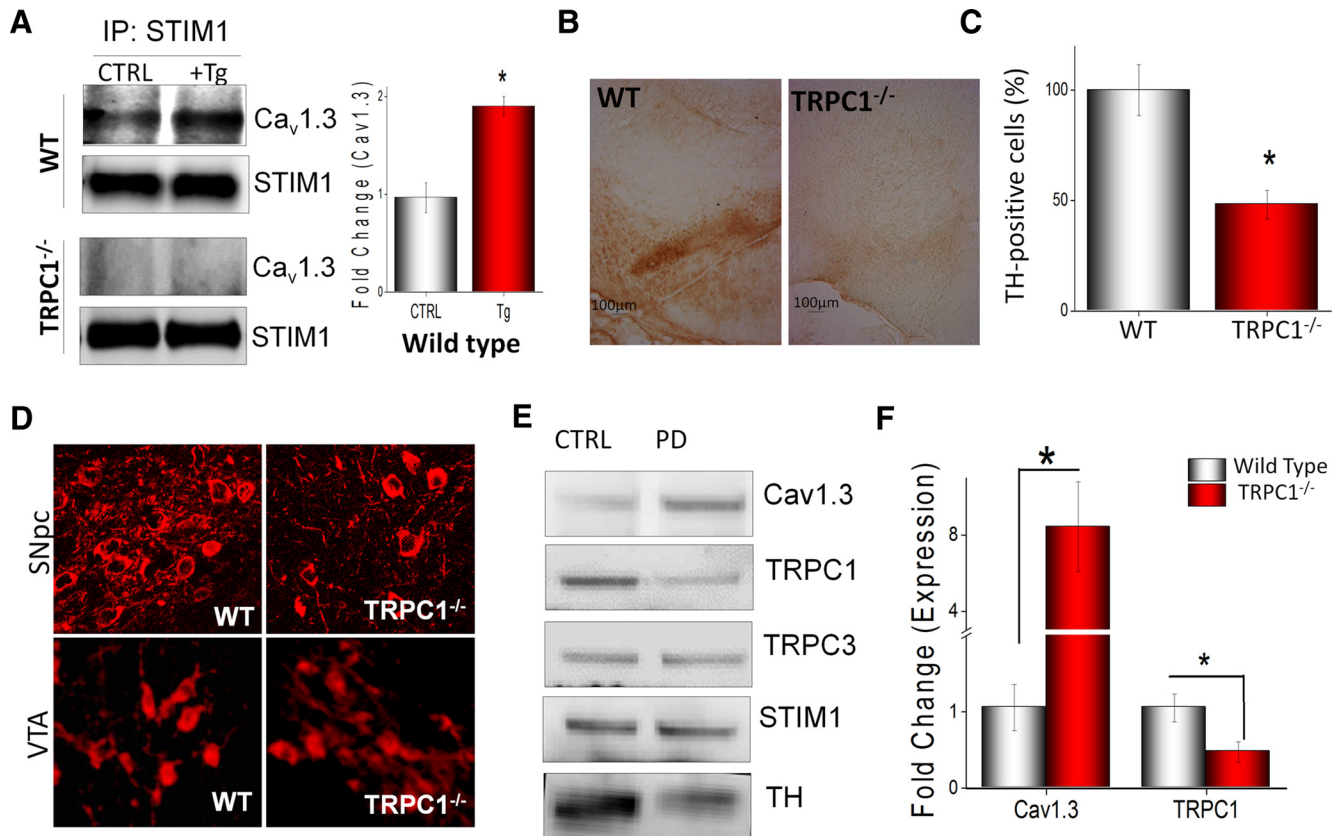
Figure 5. TRPC1 provide STIM1 scaffold that protects TH-positive neurons in SN region. **A**, Western blots of coimmunoprecipitates of SN tissues using STIM1 antibody under control (CTRL) and stimulated (store depletion, +Tg) conditions. Bar graph represents densitometry for fold change in Ca$_v$1.3 pulldown to show quantitation from at least three independent experiments. **B**, Representative DAB images of brain sections showing full SN region stained with TH antibody from wild-type and TRPC1$^{-/-}$ mice. **C**, Bar graph represents stereology of TH staining from rostral to the caudal region. Data are mean ± SE. *Significantly different values ($p < 0.05$). $n = 4$. **D**, Immunostaining of TH cells in SN and VTA region obtained from wild-type and TRPC1$^{-/-}$ mice. **E**, Western blots of lysates from SN tissues from control and PD samples. Antibodies used are labeled in the figure. **F**, Densitometric quantitation for normalized TRPC1 or Ca$_v$1.3 relative to TH expression. Data are mean ± SEM from three independent experiments. *$p < 0.05$.

decrease in TH staining, indicating that loss of TRPC1 renders SN region DA neurons more vulnerable (Fig. 5B–D). Consistent with these results, PD samples also showed a decrease in TH and TRPC1 expression without any change in STIM1 and TRPC3 expression, whereas an increase in Ca$_v$1.3 channels was observed (Fig. 5E,F). Collectively, these results indicate that inhibition of VGCC channels by TRPC1 (providing the scaffold to allow STIM1-mediated inhibition) may be the checkpoint responsible for protecting DA neurons in the SN region and loss of TRPC1.

TRPC1-STIM1 complex regulates Ca$_v$1.3 function
To further confirm the role of TRPC1 and STIM1 in regulating VGCC, we used differentiated SH-SY5Y neuroblastoma cells and evaluated SOCE activity in these cells. Importantly, store depletion (addition of Tg) showed a robust increase in the Ca^{2+} entry (SOCE) in control cells, which was significantly decreased in TRPC1-silenced cells (Fig. 6A). TRPC1 protein levels were also decreased in TRPC1-silenced cells (Fig. 6A). In addition, whole-cell recordings for VGCC were also performed, and knockdown of TRPC1 significantly facilitated VGCC activity without changing the I–V relationship (Fig. 6B,C,K). Importantly, no change in the inactivation of the VGCC currents was observed in TRPC1 silenced cells (Fig. 6D). Both the pore-forming α1-subunits of VGCC, Ca$_v$1.2, and Ca$_v$1.3 are expressed in DA cells (Sinnegger-Brauns et al., 2009; Kang et al., 2012) and are inhibited by nifedipine. To distinguish which channel is mediated by the

pharmacological effect of nifedipine in DA cells, we silenced Ca$_v$1.3 in differentiated SH-SY5Y cells. Notably, siCa$_v$1.3 decreased VGCC activity and abolished the effect of siTRPC1 on Ca^{2+} currents, indicating that TRPC1 modulates Ca$_v$1.3 activity (Fig. 6E,F). Similar results were also obtained with STIM1 silencing where decreased expression of STIM1 not only decreased STIM1 levels but also potentiated Ca$_v$1.3 activity (Fig. 6G,H). In addition, STIM1 silencing decreased store-mediated Ca^{2+} entry in SH-SY5Y cells, whereas restoration of STIM1 expression in STIM1 silenced cells (rescue) was able to restore STIM1 expression as well as store-mediated Ca^{2+} entry (Fig. 6I,J).

To establish the mechanism as to how TRPC1 modulates VGCC, we performed single-channel analysis in control and stimulated conditions (store depletion). Importantly, an increase in the single-channel activity of the L-type Ca^{2+} currents was observed, which was inhibited by the addition of nifedipine (Fig. 7A,B). Importantly, store depletion also showed a decrease in the single-channel activity of the L-type Ca^{2+} currents (Fig. 7A,C). Finally, silencing of Cav1.3 in these cells confirmed that these currents are mediated via the L-type Ca^{2+} channels, and store depletion along with silencing does not further decrease these currents (Fig. 7D,E). As TRPC1 is active upon store depletion, these results suggest that activation of TRPC1 increases STIM1 interaction with VGCC to decrease VGCC activity. To further confirm these results, we tested whether these proteins physically interact in SH-SY5Y cells. Consistent with the *in vivo* studies, immunoprecipitation using STIM1 antibodies resulted in coim-
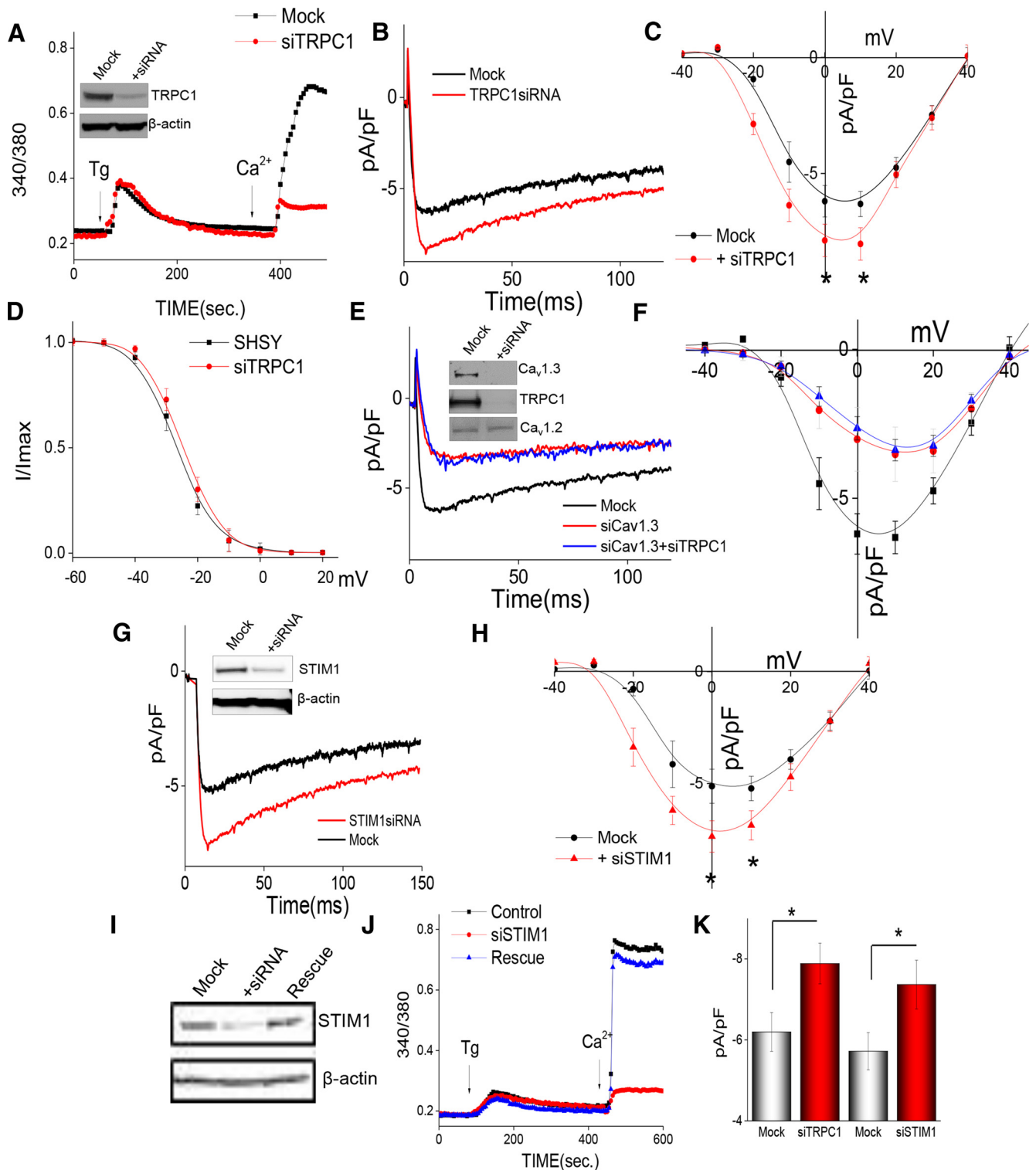
Figure 6. Ca$_v$1.3 channel activity in SH-SY5Y cells is regulated via TRPC1-STIM1. Representative Ca^{2+} traces in differentiated SH-SY5Y cells showed that silencing of STIM1 significantly inhibited Ca^{2+} influx that was induced upon store depletion (+ 1 μM Tg) (**A**). STIM1 or TRPC1 silencing increases L-type calcium currents (**B** and **G**, respectively). The average (8 –10 recordings) I–V relations under these conditions are shown in **C** and **H**, respectively (n = 9). *p < 0.05. Mean steady-state inactivation curves were obtained for L-type current in control and siTRPC1 (**D**). n = 5. Silencing of Ca$_v$1.3 (**E**, **F**) abolished the effect of siTRPRC1 on the L-type calcium current. Insets, Western blots from lysates of differentiated SH-SY5Y cells showing decreased expression of TRPC1, Ca$_v$1.3, and STIM1 (as well as overexpression of STIM1 in siSTIM1 cells, rescue) using respective siRNAs. Overexpression of full-length STIM1 in siSTIM1-expressing cells (rescue) showed an increase in Ca^{2+} influx (**I**, **J**). **K**, Average histogram for current density in various conditions. *p < 0.05.

munoprecipitation of Ca$_v$1.3 and TRPC1 (Fig. 7F). Importantly, store depletion (+Tg) showed a significant increase in Ca$_v$1.3-TRPC1-STIM1 interaction (Fig. 7F). Similar results were also obtained using Ca$_v$1.3 antibodies, which showed that Ca$_v$1.3 in-

teracts with STIM1 and TRPC1 and the association between Ca$_v$1.3-TRPC1-STIM1 was again increased upon Tg treatment (Fig. 7F). We further tested the importance of this association, and cell viability assays were performed. Importantly, TRPC1 or
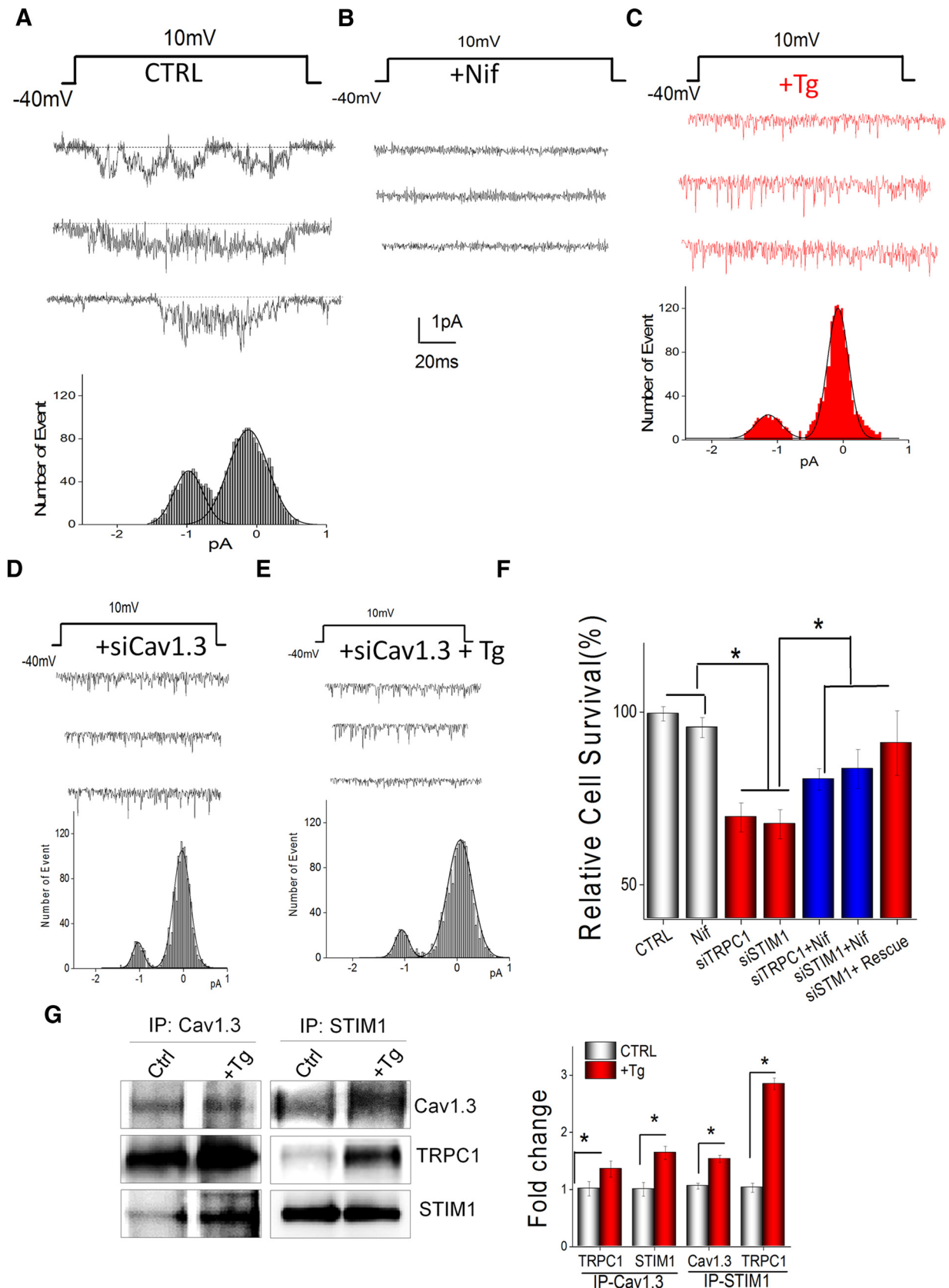
STIM1 silencing significantly decreased cell viability that was partially rescued by the addition of nifedipine (Fig. 7G). Furthermore, the rescue of STIM1 (overexpressing STIM1 after STIM1 silencing) was able to restore cell viability (Fig. 7G). Overall, these results suggest that TRPC1 decreases L-type Ca^{2+} currents by providing STIM1 scaffold, and loss of TRPC1-mediated modulation of Ca$_v$1.3 channels could lead to DA cell death.

TRPC1 regulates Ca$_v$1.3 function in neurotoxin model of PD
Neurotoxins (such as MPTP/MPP$^+$) that mimic Parkinson's disease have been shown to selectively affect the survival of DA neurons. Our previous studies have shown that MPP$^+$ treatment specifically decreased TRPC1 activity (Selvaraj et al., 2012), but the mechanism as for why these cells die was not clear. Thus, we performed electrophysiological recordings on SH-SY5Y cells with and without MPP$^+$ treatment. As indicated in Figure 5, MPP$^+$-treated cells showed an increase in Ca^{2+} currents (Fig. 8A,B). Also, the Tg-mediated inhibition of Ca^{2+} currents was abolished upon MPP$^+$ treatment (Fig. 8A,B). Importantly, overexpression of TRPC1 inhibited the MPP$^+$-induced increase in L-type calcium currents, suggesting that restoration of TRPC1 expression is essential for the inhibition of L-type calcium currents (Fig. 8A). Notably, siCa$_v$1.3 also abolished the effects of MPP$^+$ and Tg on Ca^{2+} currents, which further suggest that Ca$_v$1.3 is involved in MPP$^+$-facilitated Ca^{2+} currents (Fig. 8C,D). MPP$^+$ treatment also showed a decrease in TRPC1 expression without altering STIM1 levels, suggesting a prominent role of TRPC1 in the regulation of Ca$_v$1.3 currents (Fig. 8E,F). Consistent with these results, the association between Ca$_v$1.3-TRPC1-STIM1 was decreased in MPP$^+$-treated cells (Fig. 8G,H). To further study the role of Ca$_v$1.3 in neurotoxin models of PD as well as establish the consequence of Ca$_v$1.3-TRPC1 interactions in PD, we investigated proteins that induce cell death in the MPP$^+$/MPTP model. Here we found that pretreatment with nifedipine or siCa$_v$1.3 significantly decreased caspase 3 activations (as observed by cleaved active caspase) and Bak protein levels in the MPP$^+$ model (Fig. 8I). Furthermore, similar results were attained in the MPTP *vivo* model where subsequent increases in caspase 3, Bak, and Bid apoptotic proteins was observed. Interestingly, overexpression of TRPC1 in the SN region inhibited MPTP-induced increases in apoptotic proteins (Fig. 8J). Finally, pretreatment with nifedipine, siCa$_v$1.3, or overexpression of TRPC1 partially prevented MPP$^+$-induced cell death (Fig. 8K). Together, these data suggest that TRPC1 facilitates STIM1-Ca$_v$1.3 interactions to suppress Ca$_v$1.3 activity that inhibits caspase3 activation and decreases apoptosis, thereby protecting DA cells against neurotoxin-mediated insults that lead to PD.

Discussion
Although the mechanisms responsible for the preferential loss of DA neurons in PD have been debated for decades, loss of Ca^{2+} homeostasis could be the most important. Ca^{2+} is crucial for a broad range of neuronal functions, and regulation of intracellular Ca^{2+} concentration represents a major determinant that controls neuronal growth and survival, gene regulation, neuronal excitability, synaptic transmission, and plasticity (Berridge et al., 2000; Sippy et al., 2003; Burgoyne, 2007). Pathological neuronal insults evoke an unwanted rise in cytosolic Ca^{2+} levels, leading to Ca^{2+} overload that causes toxicity and neuronal cell death (Stout et al., 1998; Berridge et al., 2000). VGCCs are multimeric proteins that are essential for the Ca^{2+} influx in neuronal cells. Although several VGCC subunits have been identified, the pore-forming α1-subunit is the principal determinant for gating and phar-

macology. DA neurons have one of the two α1-subunits, Ca$_v$1.2 or Ca$_v$1.3, that modulate Ca^{2+} influx necessary for their function. In PD, a change has been shown in the ratio of Ca$_v$1.2 versus Ca$_v$1.3 that favors a greater utilization of Ca$_v$1.3 channels, which contributes to the function of DA SN neurons and also seems to render neurons susceptible to excitotoxicity and/or oxidative stress (Henchcliffe and Beal, 2008; Goldberg et al., 2012).

The role of the L-type Ca^{2+} channels in pacemaker activity is still controversial. Previous studies indicate that, in adult DA neurons, the pacemaking activity involves VGCC channels (especially Ca$_v$1.3) (Nedergaard et al., 1993; Mercuri et al., 1994; Chan et al., 2007; Puopolo et al., 2007; Guzman et al., 2009; Putzier et al., 2009; Drion et al., 2011). In contrast, other studies report the activity of VGCC is not necessary for firing but does contribute to slow oscillatory potentials, which will trigger Na$^+$ spikes (Guzman et al., 2009; Dragicevic et al., 2014), suggesting that for the oscillatory phenomenon underlying the pacemaking activity involves a mix of calcium and sodium channels. Although these studies indicate that Ca$_v$1.3 contributes to pacemaking activity, the mechanism of regulation and whether VGCC is essential for SN DA neurons pacemaker activity remains questionable. Importantly, Ca$_v$1.3 channels contribute to their progressive loss as observed in PD pathology (Surmeier et al., 2012; Dragicevic et al., 2015). Furthermore, blood–brain barrier-permeable VGCC blockers (e.g., isradipine) protect highly vulnerable DA SN neurons from degeneration in PD and chronic rodent models (Ritz et al., 2010; Ilijic et al., 2011), presumably by reducing Ca^{2+} overload and mitochondrial stress during pacemaking (Guzman et al., 2010; Dragicevic et al., 2015). One possibility for these discrepancies could be that other Ca$_v$1.3 activity-modulating proteins/channels could be expressed differently. In addition, Ca$_v$1.3 might cooperate with sodium channels to generate pacemaking with a degree of cooperation that is variable (Drion et al., 2011). Thus, although Ca$_v$1.3 contributes to the pacemaking activity in DA neurons, it comes with a high metabolic cost. Cytosolic Ca^{2+} levels are tightly controlled, and ATP is needed to sequester Ca^{2+} to inhibit abnormal activation of cells. Thus, dependence on Ca^{2+} for the pacemaking activity could be detrimental for DA neurons. Our results show that, in normal conditions, Ca$_v$1.3-mediated pacemaking activity is inhibited by TRPC1 thereby protecting DA neurons.

In addition to VGCC, Ca^{2+} release from intracellular ER/SR stores activates Ca^{2+} influx via the SOCE mechanism. The VGCC-mediated Ca^{2+} entry differs from SOCE as VGCCs are activated by depolarization in response to APs, whereas SOCE is activated in response to ER/SR Ca^{2+}-store depletion. Notably, both SOCE and VGCC channels are expressed in neurons (Strübing et al., 2001; Selvaraj et al., 2010; Sun et al., 2014); however, the function of SOCE channels in neuronal cells is not well defined. Members of the TRPC and Orai family have been suggested as Ca^{2+} entry channels that regulate SOCE. Additionally, STIM1 has shown to link ER Ca^{2+} store depletion and SOCE activation (Pani et al., 2008, 2012). Upon store depletion, STIM1 aggregates at ER-PM junctions that facilitate its interaction with TRPC1/Orai1 channels, thereby activating SOCE. Importantly, in PD and neurotoxin-treated samples, only TRPC1 expression was altered, whereas no change in Orai1 or STIM1 expression was observed, suggesting that TRPC1 could have a role in PD. Recent studies have also shown that STIM1 strongly suppresses VGCCs (Park et al., 2010; Wang et al., 2010; Harraz and Altier, 2014); however, nothing is known as to whether STIM1 regulates Ca$_v$1.3 channels and, if so, what mediates this interaction or what is the physiological function of this inhibition. Our results show that, in
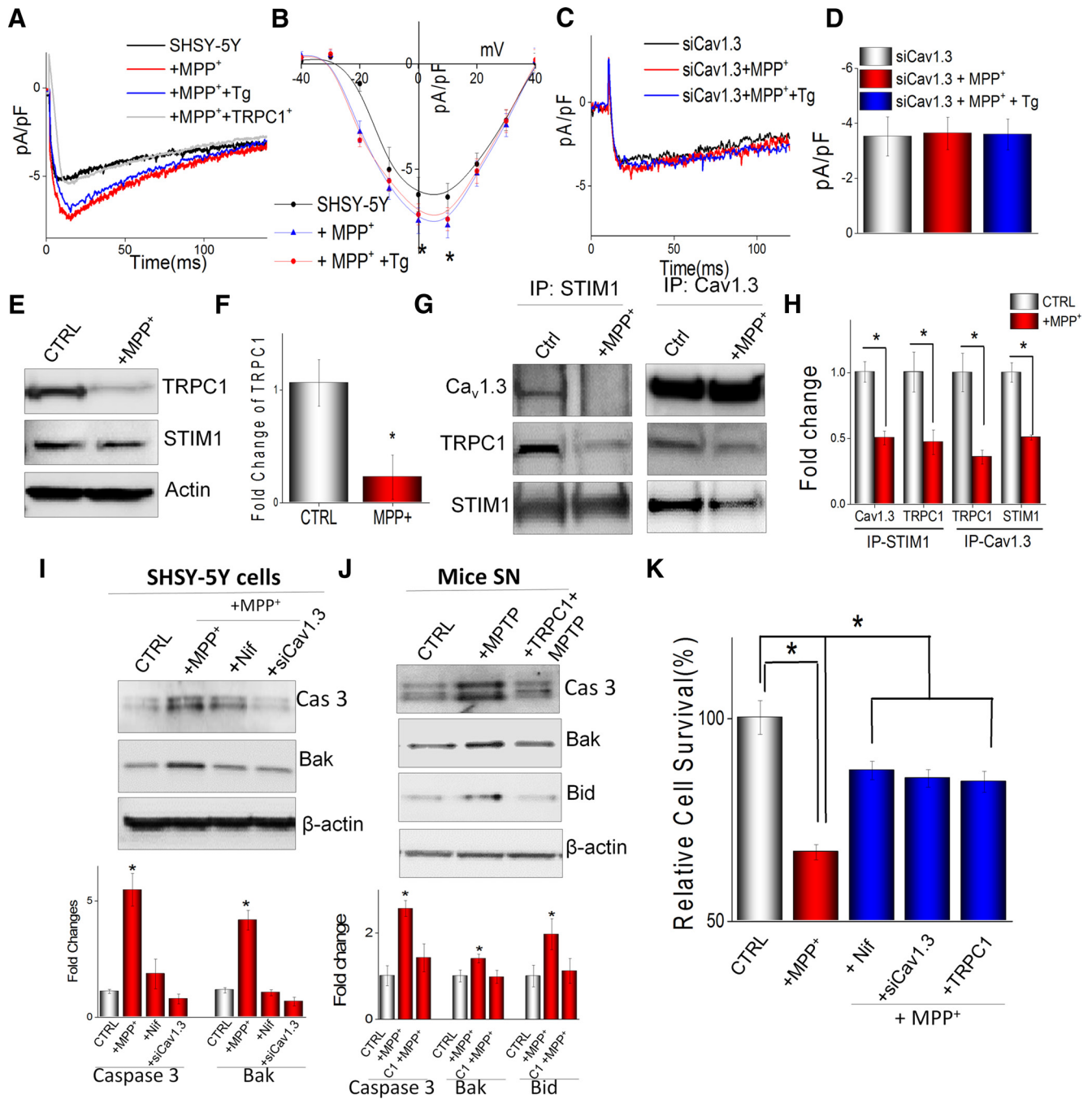
Figure 8. TRPC1 regulates Ca$_v$1.3 function in neurotoxin PD model. **A**, Representative trace showed that calcium currents were evoked in differentiated SH-SY5Y cells with a step protocol from −60 to 10 mV under various conditions. Cells treated with MPP$^+$ facilitated calcium current, but overexpression of TRPC1 abolished this effect. **B**, **I–V** curves. **E**, **F**, Western blots of lysates in control and MPP$^+$-treated SH-SY5Y cells (500 μM, 24 h). **C**, **D**, Individual traces of L-type calcium currents in Ca$_v$1.3 silenced cells with and without MPP$^+$ and/or Tg treatment. **G**, **H**, Coimmunoprecipitates of SH-SY5Y cells using Ca$_v$1.3 and STIM1 antibodies under various conditions. **I**, Western blots of lysates from differentiated SH-SY5Y cells using cleaved caspase3, Bak, and β-actin antibodies under various conditions. **J**, Western blots of lysates from mice SN region using cleaved caspase3, Bak, Bid, and β-actin antibodies under various conditions. For MPTP treatment, mice received MPTP-HCl 25 mg/kg (i.p. for 5 consecutive days at 24 h intervals). Densitometric quantitation for normalized relative to β-actin is shown below the blots. Values are mean ± SEM from three independent experiments. *$p < 0.05$. **K**, Cell viability under various conditions in SH-SY5Y cells. *Significantly different values from untreated cells ($p < 0.05$). $n = 8$.

DA neurons, STIM1 suppressed Ca$_v$1.3 channels by interacting with Ca$_v$1.3; more importantly, this association was dependent on TRPC1 expression as TRPC1 knock-out mice or TRPC1 silencing showed a significant decrease in store-dependent Ca$_v$1.3-STIM1 interaction. Moreover, store depletion increased TRPC1-STIM1 interaction and facilitated its association with Ca$_v$1.3 channels, further suggesting that TRPC1 is essential for providing the scaffold necessary for the inhibition of Ca$_v$1.3 channels.

A novel aspect of this study is the documentation of the significance of TRPC1 in regulating L-type Ca^{2+} channel, especially Ca$_v$1.3 function. Our findings reveal that TRPC1 acutely reduced Ca$_v$1.3 currents, which lead to decreased Ca^{2+} entry that could be essential for the inhibition of oxidative stress, thereby increasing the survival of DA neurons. One reason that DA neurons are more vulnerable is the abnormal activation of Ca$_v$1.3 channels, which facilitates sustained elevations in cytosolic Ca^{2+} concen-

tration (Wilson and Callaway, 2000). Increased cytosolic Ca^{2+} could induce mitochondrial dysfunction, and the resulting increase in reactive oxygen species could further advance these DA neurons toward degeneration (Dauer and Przedborski, 2003; Abou-Sleiman et al., 2006; Selvaraj et al., 2010; Sun et al., 2014). In addition to susceptibility to mitochondrial toxins, a decrease in ER Ca^{2+} levels is also known to induce ER stress, which could activate the cell death cascades, as observed in PD (Nguyen et al., 2002; Holtz and O'Malley, 2003; Yoshida et al., 2006; Sun et al., 2014). Importantly, restoration of ER Ca^{2+} stores via TRPC1 activation could protect DA cells against such conditions. Thus, activation of TRPC1 could suppress Ca$_v$1.3 channels, thus reducing mitochondrial dysfunction and alleviate ER stress to improve DA neurons survival.

Although mechanistic insights regarding how STIM1/TRPC1 may inhibit VGCC needs further investigation, elucidating the relationship between SOCE and VGCC channels in DA neurons could provide insights into the molecular cascade critical for neuronal loss. Herein, our results offer a novel mechanism where TRPC1 regulates Ca$_v$1.3 channel activity, without altering its inactivation. TRPC1 knock-out mice showed an increase in Ca$_v$1.3 channel activation and loss of TH-positive neurons, which could be due to increase in the rhythmic activity as nifedipine protected these DA neurons. In addition, TRPC1-mediated inhibition of Ca$_v$1.3 channels was dependent on STIM1 as STIM1 silencing decreased SOCE and increased Ca$_v$1.3 activity and loss of TRPC1 abolished STIM1-Ca$_v$1.3 interaction. Moreover, neurotoxins that mimic PD increased Ca$_v$1.3 channel activity, and only TRPC1 expression was affected, suggesting that TRPC1 plays an important role in neurotoxin-mediated cell death. Silencing Ca$_v$1.3 or overexpressing TRPC1 both decreased caspase3 and inhibited MPP$^+$-induced cell death. Thus, it could be suggested that TRPC1 expression facilitates STIM1-Ca$_v$1.3 interactions and is essential for the survival of DA neurons. Although TRPC1 is expressed in most neuronal cells, its loss is only observed in DA neurons in the SN region. Importantly, Ca$_v$1.3 channels are found at a significantly higher density in DA neurons in the SN region than in the VTA region. Thus, TRPC1-mediated inhibition of Ca$_v$1.3 channels is specific as it protects DA neurons by reducing the rhythmic activity in the SN region. However, loss of TRPC1 due to the presence of toxins or other insults could abnormally activate Ca$_v$1.3 channels that would render these neurons vulnerable. In essence, activity-related cellular Ca^{2+} load, mitochondrial dysfunction, as well as oxidative and metabolic stress are particularly important trigger factors for PD (Dragicevic et al., 2015). Moreover, epidemiological studies have shown an association between the use of dihydropyridines (VGCC inhibitors) and decrease the risk for PD (Ritz et al., 2010; Marras et al., 2012), which further suggests that TRPC1 activators could also be a candidate for treating PD patients. Of additional importance is that TRPC1 is also essential for inhibiting ER stress; thus, TRPC1 activation could not only inhibit Ca$_v$1.3-induced oxidative stress but also inhibit ER stress that together would protect DA neurons from the majority of stressors known to cause PD.

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