Subcellular expression and neuroprotective effects of SK channels in human dopaminergic neurons

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Small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channel activation is an emerging therapeutic approach for treatment of neurological diseases, including stroke, amyotrophic lateral sclerosis and schizophrenia. Our previous studies showed that activation of SK channels exerted neuroprotective effects through inhibition of NMDA-mediated excitotoxicity. In this study, we tested the therapeutic potential of SK channel activation of NS309 (25 \(\mu\)M) in cultured human postmitotic dopaminergic neurons in vitro conditionally immortalized and differentiated from human fetal mesencephalic cells. Quantitative RT-PCR and western blotting analysis showed that differentiated dopaminergic neurons expressed low levels of SK2 channels and high levels of SK1 and SK3 channels. Further, protein analysis of subcellular fractions revealed expression of SK2 channel subtype in mitochondrial-enriched fraction. Mitochondrial complex I inhibitor rotenone (0.5 \(\mu\)M) disrupted the dendritic network of human dopaminergic neurons and induced neuronal death. SK channel activation reduced mitochondrial membrane potential, while it preserved the dendritic network, cell viability and ATP levels after rotenone challenge. Mitochondrial dysfunction and delayed dopaminergic cell death were prevented by increasing and/or stabilizing SK channel activity. Overall, our findings show that activation of SK channels provides protective effects in human dopaminergic neurons, likely via activation of both membrane and mitochondrial SK channels. Thus, SK channels are promising therapeutic targets for neurodegenerative disorders such as Parkinson’s disease, where dopaminergic cell loss is associated with progression of the disease.

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Alterations of potassium channel expression and function in the basal ganglia have been linked to the pathogenesis of Parkinson’s disease (PD). For example, ATP-sensitive potassium (K\(_{\text{ATP}}\)) channel expression was increased upon unilateral 6-hydroxydopamine lesions of the substantia nigra pars compacta in adult rats. Activation of K\(_{\text{ATP}}\) channels by iptakalim partially alleviated haloperidol-induced catalepsy and hypolocomotion, symptoms associated with PD. Recent data showed that small-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels modulate electrophysiological properties of dopaminergic cells of the substantia nigra, where they regulate the frequency and precision of pacemaker spiking that could ultimately lead to alteration in cell survival signaling pathways. So far, neuroprotective effects of SK channels were always linked to modulation of NMDA receptors. However, we recently found that SK2 channels are also located in mitochondrial-enriched fraction, and activation of these channels prevented mitochondrial dysfunction and cell death in a neuronal cell line. Whether SK2 channels may also exert protective effects in differentiated neurons has not yet been reported. As mitochondrial dysfunction has been associated with the pathogenesis of PD, we sought to investigate this question in human dopaminergic neurons.

Lund human mesencephalic (LUHMES) cells are conditionally immortalized human fetal mesencephalic cells. After differentiation, LUHMES cells exhibit the same characteristics as human mesencephalic dopamine neurons. These cells have been validated as a model system to address neurodegenerative mechanisms related to PD. Several experimental studies showed that the mitochondrial complex I inhibitor rotenone can trigger neuronal death and cause similar pathology in animal models as observed in PD patients. Here, we addressed the question of whether activation of SK channels may exert neuroprotective activities in the human differentiated dopaminergic neurons challenged with toxic doses of rotenone.

**Abbreviations:** PD, Parkinson’s disease; K\(_{\text{ATP}}\), ATP-sensitive potassium; LUHMES, Lund human mesencephalic; DAT, dopamine transporter; VMAT-2, vesicular monoamine transporter; TH, tyrosine hydroxylase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; [Ca\(^{2+}\)]\(_i\), intracellular free Ca\(^{2+}\); ΔΨ\(_m\), mitochondrial membrane potential; PKM, PKTEN-induced kinase 1; PTP, permeability transition pore; RT, reverse transcriptase; qPCR, quantitative real-time PCR; DAPI, 4’,6-diamidino-2-phenylindole

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Results

Expression of SK channels in differentiating human dopaminergic neurons. Initial studies addressed the expression of the SK channel subtypes, that is, SK1, SK2 and SK3 in the differentiated human dopaminergic neurons. These cells are a clone of MESC2.10 cells, studied and validated earlier.14,15 The post-mitotic neuronal cells were differentiated after the v-myc transgene was switched off by tetracycline. After 4–6 days of in vitro differentiation, cells expressed the dopamine transporter (DAT), the vesicular monoamine transporter (VMAT-2), tyrosine hydroxylase (TH) and the neuronal form of β-III tubulin. Using quantitative PCR, we detected the expression of all three SK channel subtypes (KCNN1, KCNN2 and KCNN3), however, in varying quantities and expression patterns (Figure 1a). In non-differentiated state, mRNA expression analysis by quantitative PCR or reverse transcriptase (RT)-PCR showed very low levels of KCNN1/SK1 and KCNN3/SK3 channels, while KCNN2/SK2 channels were found highly expressed (Figures 1a and b). During the 6 days of differentiation, the analysis of mRNA expression revealed a progressive increase in KCNN1/SK1 and KCNN3/SK3 channel expression. However, the mRNA expression of KCNN2/SK2 channels was highly downregulated during the first day of differentiation and slowly increased after the second and third day of differentiation (Figure 1b). In addition, expression of SK channels was further confirmed by western blotting analysis (Figure 1c). These data obtained from the dopaminergic neurons 6 days differentiated from mesencephalic precursor cells in vitro with an SK3 channel subtype predominantly expressed correspond well to the expression pattern of SK channels in dopaminergic neurons of C57/BL6 mice in vivo7 and in human substantia nigra.18

Rotenone toxicity in differentiated human dopaminergic neurons. Next, we characterized the response of differentiated dopaminergic cells to the rotenone challenge. Rotenone (0–2 μM) elicited significant toxic effects in dopaminergic cells in a dose- and time-dependent manner as quantified by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and ATP measurements (Figures 2a and b). To analyze the effect of SK channel activation on rotenone toxicity, we treated dopaminergic neurons with different concentrations of NS309 (10–75 μM), a pharmacological activator for SK channels.19 At concentrations > 50 μM, NS309 induced cell death in the dopaminergic neurons (Figure 2c). Based on these results, we chose a non-toxic NS309 concentration of 25 μM for further experiments.

Activation of SK channels prevents rotenone-induced neuronal cell death and neuronal network degradation. To determine whether SK channel activation is related to neuronal survival, we tested the SK channel activator NS309 in conditions of rotenone toxicity. Dopaminergic neurons
were challenged with rotenone (0.5 μM) for 24 h in the presence or absence of NS309. Application of the SK channel activator promoted survival of dopaminergic cells challenged with rotenone, as detected by bright field microscopic analysis (Figure 3a) and after 4’-6-diamidino-2-phenylindole (DAPI) staining of the nuclei (Figure 3a). We subsequently investigated the impact of SK channel activation on the neuronal dopaminergic network by quantifying neuronal network with the image-processing program Image J with NeuriteTracer plugin.20 Using this plugin, we analyzed fluorescence microscopic images of dopaminergic neurites after immunostaining the DAT (Figure 3b). After 24 h of rotenone (0.5 μM) exposure, the dopaminergic network appeared fragmented and the neural processes degraded, developing small bead-shaped swellings (Figure 3b). Interestingly, NS309 treatment largely preserved dopaminergic network density upon rotenone treatment, suggesting that SK channels contributed to neuronal survival pathways (Figure 3b).

Quantification of the dopaminergic neuronal network revealed that rotenone induced disruption of the neuronal network, which was prevented by the SK channel activator NS309, as also detected in fluorescent microscopic images obtained after DAT immunostaining and nuclear DAPI staining (Figure 3c). Further, neuronal viability was quantified by counting the number of healthy and fragmented DAPI-stained neuronal nuclei after the exposure to rotenone in the presence or absence of NS309 (Figure 3d). MTT and cytoplasmatic ATP analysis performed after 24 h rotenone challenge confirmed that NS309 mitigated rotenone toxicity in dopaminergic neurons (Figures 3e and f). In contrast, apamin, a specific SK channel blocker,21 completely blocked the neuroprotective effect of NS309 in cultured dopaminergic cells (Figures 3e and f), suggesting that SK channel activation was essential for dopaminergic cell survival in conditions of rotenone toxicity.

Rotenone neurotoxicity is associated with reduced SK2 channel protein levels. To gain better insight into the influence of rotenone toxicity on the expression of SK channels in dopaminergic neurons, we analyzed the SK protein expression upon a challenge with toxic concentrations of rotenone. We addressed the expression of the mRNA of SK channel subtypes (KCNN1, KCNN2 and KCNN3) in the differentiated human dopaminergic neurons, using quantitative PCR (Figures 4a–c) and western blotting analysis (Figures 4d–f). Within 24 h of incubation, rotenone (0.5 μM) significantly reduced the expression of both mRNA KCNN2 and protein SK2 channels, whereas the mRNA KCNN3 and protein SK3 channel expression increased. In fact, the SK channel activator NS309 prevented the rotenone-induced decrease in SK2 channel expression (Figures 4b, c, e and f) and the rotenone-dependent increase in SK3 channel expression. Together with the survival data (Figure 3), these findings show that NS309 not only protected neurons against rotenone toxicity but also prevented rotenone-induced SK2 and SK3 channel expression changes, which could contribute to the protective effects.

Localization of SK2 channels in the mitochondrial membrane-enriched fraction. Rotenone inhibits mitochondrial complex I activity thereby activating cell death mechanisms. As activation of SK channels mitigated the detrimental effects mediated by rotenone, we investigated whether the SK channel-dependent neuroprotection correlated with preserved mitochondrial function. We isolated intact mitochondrial membrane fraction of Lühmes dopaminergic cells and analyzed protein expression of SK2 and SK3.
channels by immunoblotting. Isolation of subcellular compartments revealed an enrichment of SK2 channels from whole-cell extracts to mitochondria, suggesting that these channels reside primarily in the mitochondrial membrane of dopaminergic neurons and confirming previous reported data in hippocampal neuronal HT22 cells and in cardiac cells.23

Figure 3  Activation of SK channels prevents rotenone-induced neuronal cell death and network disintegration. Dopaminergic cells were treated with rotenone (0.5 μM) in the presence or absence of NS309 (25 μM). (a) Representative microscopic pictures and DAPI stainings depicted for dopaminergic cells untreated or treated for 24 h with rotenone (0.5 μM) and NS309 (25 μM). (b) The neuronal network was visualized by DAT immunostaining. The degree of neuronal network disintegration (depicted as Neuronal network) was analyzed using the Image J software, with the Neurite Tracer plug-in (bar graph: 20 μm). (c) The analysis of neuronal network disintegration (depicted as Neuronal network) was assessed in relation to the total area covered by DAT immunostaining and DAPI-stained nuclei. The bar graph shows the analysis of neurite tracing for n = 9–10 fields per condition, n = 3, **P < 0.01 versus non-treated dopaminergic neurons, ***P < 0.001 versus rotenone-treated dopaminergic neurons, ANOVA Scheffé’s test). (d) Nuclear damage was assessed by counting the total numbers of DAPI-positive nuclei and fragmented or pyknotic DAPI-positive nuclei. The bar graph shows the percentage of fragmented DAPI versus total DAPI staining (**P < 0.001 versus non-treated dopaminergic neurons, ***P < 0.001 versus rotenone-treated dopaminergic neurons, ANOVA Scheffé’s test, n = 500 cells per condition, n = 4). (e and f) Dopaminergic cells were challenged with rotenone (0.5 μM) in the presence and absence of NS309 (25 μM) and apamin (10–20 μM). NS309 mediated neuroprotection against rotenone toxicity as analyzed by (e) an MTT assay and (f) an ATP assay (non-significant data are shown as NS; **P < 0.01, ****P < 0.0001 versus rotenone-treated dopaminergic neurons, ANOVA Scheffé’s test, n = 6 wells per group, the experiment was repeated three times with similar results).
However, SK1 and SK3 channels were mainly localized in the cytosolic fraction (Figures 5a–c). Confocal immunofluorescence staining specific for SK2 channels showed extensive overlap between SK2 channel and mitochondrial staining in neuronal dopaminergic cells. In accordance with western blotting measurements, the confocal microscopy analysis suggests that SK2 channels and, to a lesser extent, SK3 channels are expressed in mitochondrial-enriched fractions and co-localized with the mitochondrial markers MitoTracker Deep Red (Figures 5d and e). In contrast, SK1 channels did not co-localize with mitochondrial markers in human dopaminergic cells (Figures 5d–f).

The mitochondrial localization of SK2 is further corroborated by positive predictions by the two computational methods TargetP 1.1 and Mitoprot II of a mitochondrial targeting sequence in human SK2 channels (accession number: AAP45946).

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Together, our data strongly point towards the localization of SK2 channels at the mitochondrial membrane in dopaminergic neurons, similar to previous findings in a hippocampal neuronal cell line and in cardiac cells.

SK channel activator NS309 mitigates rotenone-induced increases in \( [\text{Ca}^{2+}]_i \). Chronic elevation of basal intracellular free \( \text{Ca}^{2+} \) is a central element in diverse neuronal cell death pathways. To further study the proposed role of increased intracellular calcium levels in dopaminergic neuron cell death induced by rotenone, we performed whole-cell calcium recordings using Fluo-4 AM as a cytosolic calcium marker. NS309 did not affect the basic \( [\text{Ca}^{2+}]_i \) compared with controls in healthy cells (Figure 6a). In contrast, NS309 significantly attenuated the elevated \( [\text{Ca}^{2+}]_i \) following 24 h of the rotenone (0.5 \( \mu \text{M} \)) challenge (Figure 6a). This result shows that activation of SK channels blocked rotenone-induced increases in intracellular calcium, which are essential features of dopaminergic cell death.

For a better understanding of the neuroprotective effects mediated by activation of SK channels at the mitochondrial level, we investigated the mitochondrial membrane potential (\( \Delta \Psi_m \)) in response to SK channel activation. Isolated intact mitochondria from differentiated dopaminergic cells were exposed to the fluorescent dye DIOC6(3) for \( \Delta \Psi_m \) measurements. NS309 induced a decrease in \( \Delta \Psi_m \) under control conditions (Figure 6b).

**Figure 4** Rotenone alters the expression of SK channels. Dopaminergic cells challenged with rotenone (0.5 \( \mu \text{M} \)) in the presence and absence of NS309 (25 \( \mu \text{M} \)) for 24 h were investigated for SK channel expression. The expression of SK channel subtypes was analyzed by (a–c) qPCR (SK1 channels depicted as KCNN1, SK2 channels depicted as KCNN2 and SK3 channels depicted as KCNN3 for mRNA levels) and (d–f) western blotting. Immunodetection of whole-protein lysates probed with antibodies to SK1 (d) SK2 (e) and SK3 (f) channels and actin, as loading control. Upper panels show SK1 (d), SK2 (e) or SK3 (f) channel expression, and the lower panels show the corresponding actin expression. Densiometric analysis of western blotting bands of SK channels (depicted as ‘Integrated optical densities’ in percentage of non-treated control cells) shows that rotenone treatment induces a downregulation of SK2 channels and an upregulation of SK3 channels (non-significant data are shown as NS, \( ^* P < 0.05 \), \( ^{**} P < 0.01 \), \( ^{***} P < 0.001 \), \( ^{****} P < 0.0001 \) versus non-treated dopaminergic neurons, \( ^{#} P < 0.05 \), \( ^{##} P < 0.01 \), \( ^{###} P < 0.0001 \) versus rotenone-treated dopaminergic neurons, ANOVA Scheffé’s test, \( n = 3 \) )
mitochondria, we isolated mitochondria from mouse brain synaptosomes and from total brain and analyzed $\Delta \Psi_m$ here by Rhodamine 123 fluorescence. We performed experiments on mitochondria isolated from mouse brain synaptosomes and from total brain based on a study that showed a different susceptibility of synaptic and non-synaptic mitochondria towards calcium.\(^{26}\) However, in our experiments the synaptosomal mitochondria did not show higher susceptibility compared with non-synaptic mitochondria. Treating brain mitochondria with different concentrations of calcium, we detected a depolarization of $\Delta \Psi_m$ in the presence of extra-mitochondrial calcium (Figures 7a and b). This depolarizing effect upon NS309-calcium co-challenge was also visible, although to a lesser extent, in mouse brain synaptosomes (Figures 7c–f). These results demonstrate the activity of NS309 on mitochondrial functions not only in the immortalized human mesencephalic cell line and the derived differentiated dopaminergic neurons but also in purified mouse brain mitochondria and synaptosomes, suggesting the relevance of these activators in the brain in vivo.

Discussion

The results obtained in the present study show that the pharmacological SK channel activator NS309 provides neuroprotection to human dopaminergic neurons in a model of rotenone toxicity. Rotenone toxicity causes a sustained reduction of SK2 channel expression and an increased SK3 channel expression, changes which are prevented by NS309. Thus, increasing SK channel activity and/or maintaining SK channel expression patterns are suggested as promising therapeutic strategies of neuroprotection.

Inhibition of respiratory mitochondrial complex I by rotenone elevates basal $[\text{Ca}^{2+}]_i$ levels in dopaminergic neurons.\(^{17}\) The increase in intracellular $[\text{Ca}^{2+}]_i$ is particularly important in relation to cell death, as $[\text{Ca}^{2+}]_i$ overload activates molecular pathways leading to necrotic and apoptotic cell death.\(^{9,24,25}\) In our study, we demonstrated that activation of SK channels prevented toxic effects of rotenone, reduced rotenone-dependent $\text{Ca}^{2+}$ overload and preserved ATP production. Previous studies showed that rotenone stimulates PD pathophysiological pathways in dopaminergic neurons and mimics most clinical features of idiopathic PD in animal models.\(^{16,17,27}\) For example, rotenone mouse models for PD exhibit progressive loss of dopaminergic neurons in the nigral–striatal system.\(^{17,28}\) Furthermore, a recent clinical study suggested that rotenone was highly associated with PD symptoms in patients exposed to environmental rotenone pollution, suggesting that mitochondrial function impairment contributed to PD pathology.\(^{28}\)

The direct association between mitochondrial dysfunction and PD was demonstrated in PD patients that showed mitochondrial complex I deficiency in the substantia nigra.\(^{29}\) These findings were substantiated in PD patients with mitochondrial defects in skeletal muscle, platelets and lymphoblasts.\(^{30}\) Moreover, mutations in mitochondrial proteins, such as mutations in PTEN-induced kinase 1 (PINK1), PARK7/DJ-1, leucine-rich repeat kinase 2 and Parkin genes are involved in the development of PD. PINK1, DJ1 and Parkin have pivotal roles in mitochondrial function and turnover and free-radical metabolism. Moreover, deletion or silencing of DJ1 sensitizes cells to oxidative stress and overexpression of DJ1 mediates neuroprotection, implying a protective role for the protein.\(^{31}\) A very recent study also linked SK channels to mitochondrial proteins showing that activation of SK channels attenuated hyperexcitability in PINK1-deficient mice and in Htra2/Omi-deficient mice.\(^{32}\)

How SK channel expression and function affects dopaminergic cell survival was further investigated in this study. A possible mechanism of action for SK channel activation-dependent neuroprotection resides from similarities with pre-conditioning mechanisms that afford sustained brain tolerance as a protective response against otherwise lethal stress following transient exposure of cells to induce stress stimuli. Several lines of evidence demonstrated that the convergence point of pre-conditioning paradigms is represented by mitochondria.\(^{23}\) Hitherto, the $K_{\text{ATP}}$ channel was shown to reduce the $\Delta \Psi_m$, and this phenomenon has been proposed as the underlying mechanism of mitochondrial pre-conditioning. It has also been demonstrated that activation of potassium $K_{\text{ATP}}$ channels mediate protection in cortical and dopaminergic neurons.\(^{34,35}\) Interestingly, activation of mitochondrial calcium-induced calcium release\(^{36}\) depends on the rate at which $\text{Ca}^{2+}$ is provided to mitochondria.\(^{37}\) On the other hand, $\text{Ca}^{2+}$ accumulation in mitochondria via the uniporter is dependent on the external $\text{Ca}^{2+}$ concentration and the transmembrane potential. This is an electrophoretic process as the mitochondrial $\text{Ca}^{2+}$ entry decreases the transmembrane potential and thus the uptake rate.\(^{38}\) Moreover, the permeability transition pore (PTP) behaves as a voltage-dependent channel as high potential (~180 mV) favors the closed conformation of the PTP and depolarization increases its probability of opening.\(^{38}\) Thus, the concomitant decrease of the potential together with low-dose calcium challenge may alter the open/close probability of the PTP, which may serve as a protective means against massive mitochondrial calcium accumulation. In line with these findings, our study shows that $\Delta \Psi$ reduction was visible upon calcium co-stimulation, suggesting that reduced $\Delta \Psi_m$ may serve as an adaptive mechanism preventing from calcium overload and destruction of the organelles. Here, we showed that SK channels and, in

![Figure 5](image.png)

*SK2 channels are present in mitochondria. (a) Immunoblot analysis of whole-cell extract (depicted as ‘Cell’), cytosol supernatant (depicted as ‘Cyto’) or crude mitochondrial pellets (depicted as ‘Mito’), using antibodies against (a) SK1, (b) SK2 and (c) SK3 channels and control antibodies for protein location (COX IV—mitochondrial matrix, Tubulin—cytosol). (d) Confocal images of SK1, SK2 and SK3 channels in human dopaminergic cells. Mitochondrial staining was performed using a specific mitochondrial marker, MitoTracker Deep Red (bar graph: 5 μm). (e) Quantitative colocalization analysis of dopaminergic cells with SK (SK1/SK2/SK3) channels and MitoTracker Deep Red. (f) Image-generated scatter plots of acquired images for colocalization analysis processed by the LAS AF software (Leica). Mean percentages of colocalization rate were calculated at ROIs placed at cell body. Mean of colocalization rate: MitoTracker/SK1: 34.1%, MitoTracker/SK2: 86.1%, MitoTracker/SK3: 26.7%. Colocalization rate: This value indicates the extent of colocalization in percentage. It is calculated from the ratio of area of colocalizing fluorescence signals and the area of the image foreground (Obs. ROI: region of interest).*
particular, the SK2 channel subtype is located in neuronal mitochondria. Activation of SK channels by NS309 in intact isolated mitochondria initiated a slight decrease of $\Delta \Psi_m$. In a recent study, we demonstrated that activation of SK2 channels induced a slight depolarization of $\Delta \Psi_m$ in a hippocampal-derived HT-22 cell line. Performing patch-clamp
Figure 6  Activation of SK channels prevents calcium dysregulation. (a) Intracellular calcium was investigated in dopaminergic cells challenged with toxic concentrations of rotenone in the presence or absence of SK channel opener, NS309 (*P < 0.05, versus rotenone and NS309-treated dopaminergic neurons were considered to be significant, ANOVA Scheffé's test, n = 6). (b) Changes of the MMP (ΔΨm) were detected by DIOC6(3) fluorescence-based assay. Isolated 25–50 μg mitochondria were incubated with 20 nM DIOC6(3) dye. As a positive control for a complete loss of ΔΨm, CCCP (50 μM) protonophore was applied on intact mitochondria. ΔΨm was analyzed by a FLUOstar Optima fluorescence plate reader using 485/520-nm filters for excitation/emission.

Figure 7  ΔΨm is altered by SK channel opening. Isolated mitochondria from (a, c, e) the mouse brain and synaptosomes (b, d, f) were loaded with fluorescent dye Rhodamine 123 (depicted as Rh123), and the ΔΨm was measured. Representative Rh123 fluorescent traces of NS309 effect (25 μM) on isolated mitochondria (a and b) in the absence of extracellular calcium, (c and d) in the presence of 10 μM Ca^{2+}, and (e and f) of 40 μM Ca^{2+}. As a positive control for a complete loss of ΔΨm, FCCP (50 μM) protonophore was applied on intact mitochondria.
experiments on isolated mitoplasts, we could further show that this observation on SK channel activation-mediated slight mitochondrial depolarization was in line with a K⁺ influx via mitochondrial SK2 channels at hyperpolarized ΔΨm, evident from the currents recorded from the inner mitochondrial membrane. Under physiological conditions due to the pronounced hyperpolarized ΔΨm, at about –180 mV, there is a strong electrical driving force for a K⁺ influx. Opening of the mitochondrial SK2 channels under physiological conditions further increased K⁺ flow into the mitochondrial matrix thereby decreasing ΔΨm.15 In fact, pre-treatment with NS309 also prevented rotenone-mediated cell death in dopaminergic neurons, suggesting that opening SK channels might modify neuronal susceptibility to damaging agents to a more tolerant state thereby providing neuroprotection.

Another important finding was that NS309 preserved the expression of SK channels in dopaminergic neurons exposed to rotenone. This suggests that rotenone-induced toxicity is mediated by concomitant disruption of counteractive mechanisms such as SK channels that disappear after the toxic stimulus. In our study, rotenone particularly reduced the expression levels of the SK2 channel subtype and increased the expression levels of SK3 channel subtype in dopaminergic neurons. Recently, immune-electron microscopy established that SK2 channels are expressed exclusively in the dendrites of dopaminergic neurons, while SK3 channels are expressed in the soma and, to a lesser extent, in the dendritic neuronal network.7 Further experiments performed in subtype-specific null mice have demonstrated that SK2 channels are responsible for the precision of action potential timing, while SK3 channels contribute to action potential frequencies.7 Interestingly, in our study rotenone increased the mRNA and protein levels of SK3 channels. Together with findings on rotenone-induced enhanced dopaminergic excitability,39 our study suggests that increased SK3 channel expression and activity might be associated with increased dopaminergic excitability mediated by rotenone challenge. In line with these findings, it was previously demonstrated that the mRNA of SK3 channel is increased under neuropathological conditions and in cell stress-related events, including Alzheimer disease40 myotonic dystrophy41 or in LPS-induced microglial activation.42 Interestingly, activation of SK channels by NS309 prevented rotenone-induced SK2 channel downregulation, SK3 channel upregulation and cell death. Positive SK channel modulation using NS309,19 also decreased the responsiveness of dopaminergic neurons to depolarizing currents, enhanced spike frequency adaptation and slowed spontaneous firing, due to an increase in the amplitude and duration of after-hyperpolarization.43 These observations on SK2 channel pattern were similar to findings in models of glutamate toxicity in vitro or cerebral ischemia in vivo, where the insults induced a decline of SK2 channel expression that was also prevented by SK2 channel activators.9,44

Under pathophysiological conditions, such as cerebral ischemia, substantial increases in glutamate release alter the expression pattern of SK channels.9,44 In vivo, cerebral ischemia induced by cardiac arrest and cardiopulmonary resuscitation caused a delayed and sustained reduction of synaptic SK channel activity, as demonstrated by electrophysiological recordings. Further, immune-electron microscopy showed that reduced SK channel currents were associated with synaptic internalization of the channels.44 These findings were substantiated in primary cortical neurons exposed to toxic doses of glutamate where glutamate promoted an internalization of SK2 channel subtypes.9 Facilitation of SK2 channel function by either 1-ethyl-benzimidazolinone or NS309 preserved SK2 channel expression, reduced neuronal cell death and improved cognitive outcome.9,44

It is important to note that the SK channel activator NS309 might target both plasma membrane SK channels as well as mitochondrial SK2 channels. By targeting mitochondrial SK channels, NS309 induces a slight depolarization of the ΔΨm, thereby likely contributing to neuroprotective effects as demonstrated in the present study. On the other hand, by targeting plasma membrane SK channels, NS309 affects the firing pattern of dopaminergic neurons,43 which may also be neuroprotective.6 As the differential roles in cell survival are presently unresolved, it will be important to determine the relative contributions of plasma membrane versus mitochondrial SK channels to the protection against rotenone toxicity.

In summary, mitochondrial dysfunction and delayed cell death of human dopaminergic cells can be prevented by increasing and/or stabilizing SK channel activity. Our data suggest that positive modulation of SK channels could serve as a therapeutic strategy aiming at decreasing neuronal vulnerability triggered by mitochondrial dysfunction in conditions of cellular stress relevant to PD.

Materials and Methods

Human dopaminergic LUMHES cells. These cells are a clone of MESC2.10 cells, studied and validated previously.14,15 After 4–6 days of in vitro differentiation, cells expressed the DAT, the VMAT-2, TH, and the neuronal form of β-III tubulin.15,45 The compounds NS309, rotenone and apamin21 were obtained from Sigma, Deisenhofen, Germany. NS309 is a selective positive modulator for SK1-3/IK channels, with an absolute requirement for a minimum concentration of intracellular Ca²⁺.15 It equally changes the sensitivity of SK1, SK2 or SK3 channels towards calcium and it is devoid of any effect on BK type channels.19 NS309 was added simultaneously with the rotenone treatment.

Cell viability assessment. Quantification of cell viability was performed by the MTT reduction assay at 0.5 mg/ml for 1 h. The reaction was terminated by removing the media and freezing the plate at –80 °C for at least 1 h. DMSO solvent was added to each well for 1 h under shaking conditions at 37 °C. The absorbance of each well was determined by an automated FLUOstar Optima reader (BMG Labtechnologies GmbH, Inc., Offenburg, Germany) at 570 nm with a reference filter at 630 nm. Dopaminergic cell death was also assessed by analysis of nuclear condensation/fragmentation after staining with 1 μg/ml of DAPI (Sigma-Aldrich, München, Germany). Dopaminergic neuronal nuclei were considered healthy when they showed moderate and homogenous fluorescence. Damaged nuclei were identified by condensed chromatin gathered around the nuclear membrane, bright fluorescence of pyknotic nuclei or as total fragmented nuclear bodies. More than 500 cells were counted per condition (n = 4), and the percentage of apoptotic nuclei was determined. The experiment was repeated with three independent sets of differentiated neurons, and counting was performed blinded to the experimental condition.

ATP measurements. Human differentiated dopaminergic cells were seeded in white 96-well plates (Greiner bio one, Frickenhausen, Germany) for luminescence measurements. Following differentiation (days 5–6) dopaminergic cells were challenged with rotenone (0.5 μM). ATP levels were detected at 24–48 h after the onset of rotenone exposure by detection of luminescence using the ViaLight MDA Plus-Kit (Lonza, Verviers, Belgium). The cells were treated first with a ‘nucleotide-releasing reagent’ (100 μl/well) and incubated for 5 min at room temperature. Afterwards the ATP

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monitoring reagent' was injected into each well (20 \mu M), and luminescence was detected immediately (Fluostar, BMG Labtech, Offenbach, Germany). The emitted light intensity was measured for quantification of ATP levels. The values of control baselines at 24 and 48 h time interval showed no significant difference.

**RT-PCR.** Total RNA was extracted using the NucleoSpin RNA II kit (MACHEREY-NAGEL GmbH and Co. KG, Düren, Germany) following the manufacturer's instructions. RT reactions were conducted using the SuperScript III One-Step RT-PCR System (Invitrogen, Karlsruhe, Germany) in a SensoQuest Lab Cycler (SensoQuest Biomedizinische Elektronik GmbH, Göttingen, Germany). The following amplification of cDNA by PCR using specific primers was carried out by the following steps: (1) denaturing at 95 °C for 4 min; (2) 94 °C for 30 s; (3) \( T_m \) (annealing temperature) for 30 s, depending on the SK channel isoform of interest; (4) extension at 72 °C for 30 s; with 30 cycles from steps 2–4. The final extension step was set at 72 °C for 5 min. The \( T_m \) for SK1 channels was 63 °C, 57.3 °C for SK2 channels and 61 °C for SK3 channels.

**Calcium measurements.** To study the effects of rotenone on \([Ca^{2+}]_i\), we used a Fluostar Optima system (BMG Lab Technology, Göttingen, Germany). Dopaminergic cells grown on black 96-well plates (15 000–17 000 cells/well) were loaded for 30 min with 2 \mu M Fluo-4 AM (Molecular Probes, Eugene, OR, USA) in a solution containing 0.005% pluronic acid and 2 \mu M probenecid (Invitrogen). The excitation/emission filter pair 485/520 nm was used in the experiments.

**Immunocytochemistry.** Dopaminergic cells were fixed using PFA 4% and permeabilized using Triton X 0.04%. Incubation with primary antibodies against DAT at a concentration of 1:100 was conducted overnight at 4 °C, followed by secondary anti-rabbit antibodies coupled to DyLight 649 (Invitrogen). Images were acquired using an epifluorescent microscope (DMI 6000 B, Leica, Wetzlar, Germany) connected to a CCD camera (DFC 360 FX, Leica). Light was acquired using an epifluorescent microscope (DMI 6000 B, Leica, Wetzlar, Germany). The membranes were incubated overnight with primary antibodies (1:3000; rabbit anti-SK2 channel and 1:1000 rabbit anti-SK3 channel) at 4 °C, washed and analyzed with Leica Application Suite Advanced Fluorescence (LAS AF Version 2.6.3.8173; Leica Microsystems CMS, Wetzlar, Germany).

**Neurite measurements.** Neuronal networks were analyzed using the Image J program with Neurite Tracer Plug-in. Neuronal network damage has been recognized to be a key predictor of outcome in a number of diverse human CNS diseases. The nucleus was stained with 1 \mu M DAPI and the neuronal dendrites with antibodies against DAT at a concentration of 1:100. Images were acquired using an epifluorescent microscope (DMI 6000 B, Leica) connected to a CCD camera (DFC 360 FX, Leica). The analysis was performed according to Pool et al. Briefly, the analysis was performed on pairs of images corresponding to nuclear (DAPI staining) and neurite marker (DAT staining) images. Image acquisition was performed using the same settings of illumination and contrast. The analysis was carried out by subtraction of a background image to reduce artifacts generated by the acquisition system as well as contrast enhancement, rolling ball radius background subtraction. These pre-processing steps provided the threshold and image scale, which were applied to all the processed images. Later, small spots were automatically removed using the 'Particle Remover' plug-in. Although the neuronal stack was skeletonized, parts of the skeleton corresponding to the cell soma were removed by subtracting them automatically from the neuronal nuclei stack. The area covered by the processed skeleton in each image was measured as an estimate of the total length of the neuronal network. Algorithms used in Neurite Tracer plugin detect pixels in line segments by local geometric properties of the lines, such as ridges and ravines. This plugin utilizes the Gaussian smoothing kernel that effectively extracts line pixels by using the first and second derivatives of the line pixels. The advantage of these algorithms is their ability of dealing with uneven intensity images. However, a major limitation of Neurite Tracer plugin consists in the fact that it does not distinguish attachment points, ending points and neurites located in close proximity or in bundles.

**RT quantitative PCR.** For RT quantitative real-time PCR (qPCR) analysis, mRNA was extracted with the NucleoSpin RNA Kit (MACHEREY-NAGEL). mRNA concentration was measured by Nanodrop2000 (Thermoscientific, Bonn, Germany) and 1000 ng mRNA was reversely transcribed into cDNA with the Script \\text{cDNA synthesis kit} (ChemDoc XRS Bio-Rad, Karlsruhe, Germany). In all, 25 ng of cDNA was used as the template for qPCR. All qPCRs were run with Tagman Universal Master Mix II with UNG (Applied Biosystems, Life Technologies GmbH, Darmstadt, Germany) and analyzed with Stepone software v 2.2.2 (Applied Biosystems). The threshold cycles (Ct) were determined for each gene, and gene expression levels were calculated as relative expression compared with the housekeeping genes.

**Mitochondrial fractionation.** Dopaminergic cells were lysed in 250 mM Sucrose, 20 mM HEPES, 3 mM EDTA, complete mini protease inhibitor cocktail tablet and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich). Cell lysates and crude mitochondria were prepared from cultured dopaminergic cells as previously described. Immunoblotting was performed with commercially available antibodies: anti-actin (Abcam ab8227, Cambridge, UK) and anti-COX IV (Abcam ab16056).

**Isolation of rat brain mitochondria.** Mitochondria were isolated by differential centrifugation according to the standard protocols. Briefly, freshly removed brain tissue was homogenized with a glass Teflon homogenizer in isolation buffer (10 mM triethanolamine (TEA), 10 mM acetic acid (HAc), 280 mM sucrose, 0.2 mM EGTA, pH 7.4 with KOH). Homogenates were cleared from debris and nuclei by two centrifugation at 750 \times g (10 min at 4 °C), and mitochondria were pelleted at 9000 \times g (10 min at 4 °C). Organelles were washed three times (once at 9000 \times g and two times at 15 000 \times g, 10 min, 4 °C) and resuspended in FFE separation buffer (10 mM TEA, 10 mM HAc, 280 mM sucrose, pH 7.4 with KOH).

**ΔΨm measurements.** Changes of the \( Ψ_m \) in human dopaminergic cells were detected and quantified by DIOC6(3) fluorescence-based assay. \( Ψ_m \) in synaptosome and brain mitochondria was performed with Rhodamine 123 fluorescence-based assay. In mitochondria, the lipophilic Rhodamine-123 fluorescence dye is quenched and accumulates in the mitochondrial matrix. The higher the dissipation of \( Ψ_m \), the more Rhodamine 123 is taken up into the matrix and the \( Ψ_m \) depolarization leads to increased fluorescence as Rhodamine 123 unquenches from the mitochondria into the cytoplasm. In all, 50–100 \mu g mitochondria was incubated with the lipophilic dye 20 mM DIOC6(3) and treated with NS309 (25 \mu M). At the end of the experiment, as a positive control for a complete loss of \( Ψ_m \), CCCP (10 \mu M) or FCCP protonsophene was applied on intact mitochondria. \( Ψ_m \) was analyzed by a FLUOSTar Optima fluorescence plate reader (BMG Labechnicles GmbH, Inc.). Measurements were performed in triplicate and are representative of at least three independent experiments.

**Western blotting.** Dopaminergic neurons were lysed in 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X, pH 7.4, 78.75 °C, 0.2 mM protease inhibitor cocktail tablet and phosphatase inhibitor cocktail 1 and 2 (Sigma-Aldrich). The membranes were incubated overnight with primary antibodies (1:1 000; rabbit anti-SK2 channel and 1:1000 rabbit anti-SK3 channel) at 4 °C and afterwards with peroxidase-conjugated secondary antibodies (1:2500).

**Statistical analysis.** All data are given as means ± S.D. For statistical comparisons between the two groups, Student's t-test was used; multiple comparisons were performed by ANOVA followed by Scheffe's post hoc test. Calculations were made with the Winstat standard statistical software package (Robert Fitch Software, Bad Krozingen, Germany). A statistically significant difference was assumed at \( P<0.05 \), **\( P<0.01 \) or ***\( P<0.001 \).

**Conflict of Interest**

PC is a full-time employee at Aniona. The remaining authors declare no conflict of interest.

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**Author Contributions**

AMD, AA and LM carried out experiments and performed data analysis. AMD, NP, GH and CC participated in the design and coordination of the study and edited the manuscript. H-G K, MH, PC and HZ provided insightful discussions and designed parts of the study. AMD and CC wrote the paper. All authors read and approved the final manuscript.
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