Targeting PERK signaling with the small molecule GSK2606414 prevents neurodegeneration in a model of Parkinson's disease

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1. Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder, leading to the progressive decline of motor control due to the loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Accumulating evidence suggest that altered proteostasis is a salient feature of PD, highlighting perturbations to the endoplasmic reticulum (ER), the main compartment involved in protein folding and secretion. PERK is a central ER stress sensor that enforces adaptive programs to recover homeostasis through a block of protein translation and the induction of the transcription factor ATF4. In addition, chronic PERK signaling results in apoptosis induction and neuronal dysfunction due to the repression in the translation of synaptic proteins. Here we confirmed the activation of PERK signaling in postmortem brain tissue derived from PD patients and three different rodent models of the disease. Pharmacological targeting of PERK by the oral administration of GSK2606414 demonstrated efficient inhibition of the pathway in the SNpc after experimental ER stress stimulation. GSK2606414 protected nigral-dopaminergic neurons against a PD-inducing neurotoxin, improving motor performance. The neuroprotective effects of PERK inhibition were accompanied by an increase in dopamine levels and the expression of synaptic proteins. However, GSK2606414 treated animals developed secondary effects possibly related to pancreatic toxicity. This study suggests that strategies to attenuate ER stress levels may be effective to reduce neurodegeneration in PD.
Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Prion-related disorders (PrDs), Huntington's disease among others (Bertram and Tanzi, 2005; Soto, 2003). Although the neuronal populations and the clinical manifestation of specific PMDs are diverse, most of these conditions share the occurrence of proteostasis alterations which may underlay the occurrence of protein misfolding and neuronal dysfunction (Giechansow and Kwon, 2017; Hetz and Saxena, 2017; Kaushik and Cuervo, 2015).

Emerging evidence suggests that a reduction in the buffering capacity of the proteostasis network during aging operates as risk factor to undergo neurodegeneration (Kaushik and Cuervo, 2015). One of the nodes of the proteostasis network more affected in PD involves dysfunction of the folding capacity of the endoplasmic reticulum (ER) (Michel et al., 2016). In fact, ER stress is a common feature of PD as reported in patient derived post-mortem brain tissue (Conn et al., 2004; Hoozemans et al., 2007; Selvaraj et al., 2012; Siodzinski et al., 2009) and various animal and cellular models of the disease (reviewed in Mercado et al., 2016). ER stress was also shown to be a major pro-survival mechanism in stem cell derived dopaminergic neurons from PD patients (Chung et al., 2013; Heman-Ackah et al., 2017). At the molecular level, the induction of chronic ER stress by α-Synuclein has been related to a direct inhibition of the vesicular trafficking between ER and Golgi (Cooper et al., 2006; Gitler et al., 2008; Su et al., 2010; Thayanidhi et al., 2010). In addition, its accumulation inside the ER results in abnormal interactions with BiP (Bellucci et al., 2011; Colla et al., 2012a; Colla et al., 2012b), which may alter protein folding and maturation. ER stress has been also linked to other PD genes, including LRRK2, Parkin, Pael-R, DJ-1, and ATP13A2 (Mercado et al., 2016; Tsuji et al., 2015). Altogether, several studies delineate a paradigm where proteostasis defects and abnormal protein accumulation during aging results in neuronal loss and synaptic dysfunction in PD. Importantly, ER stress is also emerging as a common and transversal pathological mechanism of most frequent neurodegenerative diseases (Hetz and Saxena, 2017; Schepers and Hoozemans, 2015; Smith and Malucci, 2016).

To alleviate ER stress, cells activate the unfolded protein response (UPR), an integrated signaling pathway that aims to re-establish proteostasis (Hetz, 2012; Walter and Ron, 2011). However, chronic or irreversible ER stress can lead to cell death by apoptosis (Tabas and Ron, 2011; Urra et al., 2013). The UPR is mediated by three main stress sensors known as Inositol requiring enzyme alpha (IRE1α), RNA-like ER kinase (PERK) and activating of transcription-6 (ATF6). PERK is a type-I transmembrane kinase located at the ER membrane controlling cell fate under ER stress (Hetz and Papa, 2017). Activation of PERK leads to the phosphorylation of the eukaryotic translation initiation factor eIF2α, inhibiting general protein synthesis (Harding et al., 1999). This event quickly reduces the load of proteins entering the ER, having an important pro-survival role (Harding et al., 2000). Phosphorylation of eIF2α allows the selective translation of certain mRNAs containing short open reading frames in their 5′-untranslated regions, highlighting the mRNA encoding for activating of transcription-4 (ATF4) as a major substrate (Harding et al., 2000). ATF4 controls the expression of a cluster of genes involved in redox control, amino acid metabolism, macroautophagy, protein synthesis and folding (Han et al., 2013; Harding et al., 2003). Under prolonged or chronic stress, ATF4 also regulates the expression of pro-apoptotic genes such as the C/EBP-homologous protein (CHOP, also known as GADD153), tribbles-related protein 3 (TBR3) and members of the BCL-2 protein family (Ma et al., 2002; Ohoka et al., 2005; Zinszner et al., 1998). The levels of eIF2α phosphorylation are negatively controlled by two phosphatase complexes, the ER stress inducible form composed by PPP1R15A/GADD34 (a target of ATF4/CHOP) and the constitutive phosphatase PPP1R15B/CreP (Tsaytler and Bertolotti, 2013). Dephosphorylation of eIF2α may burst protein synthesis on stressed cells, inducing oxidative stress and proteotoxicity (Marciniak et al., 2004; Zinszner et al., 1998). Importantly, in addition to PERK, eIF2α phosphorylation is a convergent point of several stress pathways mediated by the kinases GCN2, PKR and HRI, known as the integrated stress response (ISR) (Pakos-Zezbrucka et al., 2016).

The involvement of ER stress in neurodegenerative diseases is complex and may depend on the specific outputs controlled by the UPR. Extensive studies have linked PERK signaling with PMDs and other pathological conditions affecting the nervous system. For example, CHOP expression was reported as a factor mediating dopaminergic neuron loss in toxicological models of PD in mice (Silva et al., 2005). ATF4 overexpression at the SNpc using adeno-associated viruses (AAV) also reduced dopaminergic neuron survival (Gully et al., 2016). Similarly, PERK inhibition was shown to protect against mutant Pink1 and Parkin in fly models (Celardo et al., 2016). In contrast, the ATF6 and IRE1α pathways have been proposed to be mostly neuroprotective in the context of experimental PD (Credie et al., 2015; Egawa et al., 2011; Sado et al., 2009; Valdes et al., 2014). Studies in ALS models showed that PERK haploinsufficiency exacerbates the progression of the disease (Wang et al., 2011). Consistent with this, treatment of animals with inhibitors of eIF2α phosphatases, or the genetic disruption of GADD34, delay ALS progression (Hetz and Saxena, 2017). In contrast, Atf4 deficiency protected against ALS possible due to a reduction in the levels of apoptosis (Matus et al., 2013), suggesting a dual role of the pathway in this specific disease. In models of multiple sclerosis (Clayton and Popko, 2015) and spinal cord injury (Valenzuela et al., 2012; Valenzuela et al., 2016b), PERK signaling operates as an essential survival factor of oligodendrocytes, potentiating locomotor recovery (Clayton and Popko, 2016). Similar results were reported in models of Charcot–Marie–Tooth disease, where Chop deficiency or inhibition of eIF2α phosphatases improves motor function involving protection of Schwann cells (Das et al., 2015; Pennuto et al., 2008; Sidoli et al., 2016). All together, these studies indicate that eIF2α phosphorylation is protective in models of neurodegeneration, possibly by reducing the levels of ER stress and abnormal protein aggregation, whereas ATF4/CHOP expression triggers neuronal apoptosis during late disease stages.

Recent findings revealed a new pathological role of PERK signaling in neurodegenerative diseases, where sustained translational repression has adverse consequences to synaptic function (Mercado and Hetz, 2017). Studies in models of PrDs indicated that chronic eIF2α phosphorylation represses the synthesis of essential synaptic proteins, leading to behavioral impairment (Moreno et al., 2012). A small molecule that inhibits PERK known as GSK2606414 was identified through structure-guided lead optimization (Axt et al., 2012). GSK2606414 is orally active (Moreno et al., 2013) and effective in cancer models (Atkins et al., 2013; Axt et al., 2012). Administration of GSK2606414 prevented translational repression on mouse models of PrDs and Tauopathies, providing strong neuroprotection (Moreno et al., 2013; Radford et al., 2015). However, another study suggested that PERK activation protects against Tau pathogenesis (Bruch et al., 2017). In the context of experimental AD, genetic ablation of Perk expression in the brain improved neuronal physiology and reduced cognitive decline (Ma et al., 2013). In addition, local expression of ATF4 in the axonal compartment was shown to trigger neurodegenerative cascade that propagates in a cell-autonomous manner (Baleriola et al., 2014). These observations are consistent with the observation that the control of protein synthesis at the level of eIF2α phosphorylation by the ISR is essential to fine-tune neuronal plasticity (reviewed in (Buffington et al., 2014)). In summary, all these reports suggest a complex scenario where, depending on the disease context, PERK signaling may have contrasting and even opposite effects, impacting neuronal survival/ apoptosis, stress mitigation, and synaptic function.

In the current study, we investigated the possible contribution of PERK to dopaminergic neuron loss. Analysis of human PD postmortem brain tissue, in addition to the brain of three different rodent models of the disease, indicated a clear molecular signature associated with PERK/ATF4 activation. We then explored the possible neuroprotective effects of the oral administration of GSK2606414 in a mouse model of...
PD. We confirmed that this route of drug delivery inhibits the target at the SNpc when animals were exposed to the classical ER stress-inducing agent tunicamycin. Using a pharmacological model of PD, we demonstrated that blocking PERK signaling significantly protects nigral-dopaminergic neurons, improving motor performance. Importantly, GSK2606414 administration elevated the levels of dopamine and its metabolite DOPAC, in addition to restoring the levels of the synaptic proteins SNAP25 and VAMP2 in the striatum of animals exposed to the PD-inducing neurotoxin. Our results suggest that strategies to reduce chronic ER stress signals emerging from PERK may have important effects in improving dopaminergic neuron function and survival in PD patients.

2. Materials and methods

2.1. PD human tissue

Human brain specimens were obtained from the Biobank Pathology unit, VU University Medical Center under approval of the ethics committee of the VU University Medical Center. Human autopsy material was obtained with informed consent for research. Neuropathological diagnosis was performed using immunohistochemical stainings for Amyloidβ, Tau, α-Synuclein, TDP-43 and p62/SQSTM1. Analysis of formalin-fixed and paraffin-embedded tissue from different parts of the brain was performed, including the frontal cortex (F2), temporal pole cortex, parietal cortex (superior and inferior lobule), occipital pole cortex including BA17 and 18, amygdala and the hippocampus, essentially CA1 and entorhinal area of the parahippocampal gyrus.

2.2. Animal treatments

Three months-old wild-type C57BL/6 mice and female adult Sprague–Dawley rats weighing approximately 200 g were used. In addition to transgenic mice expressing mutant α-Synuclein mutant A53T (aSynG53Ttg) previously described in (Gliasson et al., 2002) and obtained from the Jackson’s laboratory, USA. All animals were housed in a 12 h light/dark cycle, with ad libitum access to food and water. All experiments were performed in accordance with the guidelines set by the animal care and use committee of the Faculty of Medicine at the University of Chile, with approved animal experimentation protocols CBA#0717 and CBA#0658 FUMUCH. GSK2606414 (GlaxoSmithKline, USA) was formulated in the vehicle (0.5% HPMC and 0.1% Tween-80 in water, pH 4.0) as a suspension and administrated by oral gavage at final concentration of 100 mg/kg/day. In the case of mice treatments, oral gavage was applied twice a day [50 mg/kg]. And in the case of rats, oral gavage was applied once a day [100 mg/kg].

2.3. Pharmacokinetics

GSK2606414 concentrations were quantified in serum (1:1–1:4 dilution with water) and brain tissue homogenates (frontal cortex, 1:4 dilution with 20:80 methanol:water) at Alliance Pharma (Malvern, PA, USA) by protein precipitation followed by HPLC-MS/MS. The lower limit of quantification of GSK2606414 was 1.0 ng/mL.

2.4. Pharmacological stimulation of ER stress

To determine the efficacy of GSK2606414 in vivo injections with the ER stress agent tunicamycin were performed directly into the SNpc using brain stereotaxis after a pretreatment with GSK2606414. Tunicamycin injections [5 mg/mL] were performed by injecting 2 μL in the SN using a 5 μL Hamilton syringe (Hamilton, USA) at the following coordinates for mice: antero-posterior (AP): +0.29 cm, medio-lateral (ML): −0.13 cm relative to bregma and dorso-ventral (DV): −0.42 cm relative to skull surface (according to the atlas of Franklin and Paxinos Second Edition, 2013). For rats, injections were performed following coordinates: AP: −0.52 cm, MD: −0.2 cm relative to bregma DV: −0.78 cm relative to skull surface (according to the atlas of Paxinos and Watson Seventh Edition, 2014). Injections were conducted at a rate of 0.5 μL/min and the needle was left in place for 5 min before retraction. Animals were euthanized 24 h after tunicamycin injections for biochemical analysis.

2.5. Genetic model of PD in rats

Recombinant AAV vectors were produced using the plasmid kindly provided by Dr. Aebersch team (Coune et al., 2011). Purification of AAV particles and titration were performed as described before (Valenzuela et al., 2012). Two μL of viral preparation (1.5 × 109 VGs/mL) were injected in the right brain hemisphere of female adult Sprague–Dawley rats using a 5 μL Hamilton syringe at a speed of 0.2 μL/min. The needle was left in place for an additional 5 min before being slowly withdrawn. The SNpc was targeted at the following coordinates: AP: −0.52 cm, MD: −0.2 cm relative to bregma DV: −0.78 cm relative to skull surface (according to the atlas of Paxinos and Watson Seventh Edition, 2014). Rats were euthanized 3 months after surgical procedure for biochemical analysis.

2.6. Toxicological model of PD in mice

6-OHDA (Sigma-Aldrich, USA) was dissolved fresh at a concentration of 4 μg/μL in 0.2% ascorbic acid (Sigma-Aldrich, USA). Injections of 6-OHDA were performed in a single point, injecting 2 μL in the right striatum using a 5 μL Hamilton syringe (Hamilton, USA) at the following coordinates: AP: +0.07 cm, ML: −0.17 cm relative to bregma and DV: −0.31 cm relative to skull surface (according to the atlas of Franklin and Paxinos Second Edition, 2013). The injection was conducted at a rate of 0.5 μL/min and the needle was left in place for 5 min before needle retraction. Mice were euthanized 21 days after the surgical procedure for histological and biochemical analysis.

2.7. Motor behavioral studies in mice

All motor tests were performed 7 days before the injection of 6-OHDA (base line) and then 7, 14 and 21 days after injections until animal’s euthanasia. The cylinder test was used to evaluate spontaneous forepaw akinesia associated to 6-OHDA hemi-lesions as we reported (Castillo et al., 2015). Animals were placed in a cylinder of transparent acrylic of 10 cm in diameter and 20 cm high and left to explore freely. The camera was placed above the cylinder and movements were
recorded for 5 min. The analysis was performed blind. The results were plotted as a percentage of touches with left forepaw (contralateral to injection side) respect to total of number touches with both forepaws. For the rotarod assay, mice were positioned on a rotating wheel (Model LE8500, Panlab SL) oriented horizontally using acceleration mode, where the speed of rotating wheel start with 4 rpm and increases to 40 rpm over 2 min. The test was performed 5 times in one day and the time of latency to fall recorded. The results were plotted as percentage of base line performance. Hanging test was performed as we recently reported (Woehlbier et al., 2016). In brief, individual mice were placed hanging with their forepaws on a 39 cm length horizontal wire and 35 cm from the base. The reaction of the mouse and the body position was observed for 30 s and recorded with a video-camera. The test was performed 3 times in one day. A score was derived from each trial. A score of 0 was given when the mouse could not hold onto the bar for > 10 s, a score of 1 when the mouse maintain itself on the wire with the forepaws, a score of 2 if the mouse maintain itself with the forepaws and tried to use its hind paws but without success, a score of 3 if the mouse used forepaws and one or two of the hind paws, a score of 4 if the mouse used all four paws and the tail and 5 if the mouse actively escapes of the horizontal wire up to the vertical support and 6 if the mouse is able to down the vertical support to the base in < 30 s.

2.8. Quantification of biogenic amines

Mice injected with 6-OHDA and treated with vehicle or GSK2606414 (100 mg/kg/day) were euthanized after 21 days. Brains were rapidly removed and the striatum dissected. Striatal tissues were stored at ~80 °C. The tissue extracts were then analyzed using Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC-MSMS) in the Biomarkers Core Lab at Columbia University Medical Center. The tissue homogenate was spiked with the internal standard cocktail, and the protein was precipitated with 0.3 M perchloric acid and centrifuged at 12,000 g for 5 min. The supernatant was mixed with 1 M sodium bicarbonate and 1% dansyl chloride, vortexed and heated at 60 °C for 10 min. After chilled on ice for 2 min, salt was precipitated with 400 μL of acetonitrile, centrifuged at 12,000 g for 5 min and the upper phase was mixed with 400 μL of ethyl acetate. The supernatant after centrifugation was evaporated to dryness under a nitrogen stream and suspended in 30 μL acetonitrile for LCMS analysis. LCMS analysis was carried out on a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA, USA). The system was controlled by MassLynx Software 4.1. Five microliters of the sample was loaded onto a Waters ACQUITY UPLC BEH Phenyl column (3 × 100 mm, 1.7 μm), maintained at 40 °C. The flow rate was 300 μL/min. The initial conditions were 50% phase A (water containing 0.1% formic acid) and 50% mobile phase B (acetonitrile containing 0.1% formic acid). Solvent B was increased linearly to 99% over 5 min and maintained till 7.5 min with a total run time of 10 min. Positive ESI-MS/MS with multiple reaction monitoring (MRM) mode was performed using the following parameters: capillary voltage 4 kV, source temperature 150 °C, desolvation temperature 500 °C, desolvation gas flow 1000 L/h. Concentrations of biogenic amines were quantitated by comparing integrated peak areas for those of each species against those of known amounts of purified standards.

2.9. Tissue preparation for biochemical analysis

Animals were sacrificed by CO2 asphyxiation, brains were removed and the ventral midbrain (containing entire SNpc), striatum, and cortex from both hemispheres were rapidly dissected on the surface of an ice-cold plastic dish. The tissue was homogenized on 100 μL of ice-cold 0.1 M phosphate buffer saline (PBS) (pH 7.4) supplemented protease inhibitor and phosphatase inhibitor cocktails (Roche applied science, USA). The homogenate was divided in two fractions for preparing total mRNA and protein extracts followed by standard methods and quantification protocols. Protein extraction was performed in TEN buffer (10 mM Tris pH 8.0; 1 mM EDTA; 0.1 M NaCl) containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche applied science, USA) followed by sonication. Sample quantification was performed with micro-BCA assay kit (Pierce, USA). Antibodies and dilutions used are the following: β-actin (1:20,000, MP Biomedicals, USA), ATF4 (1:2,000, Santa Cruz, USA), α-Synuclein (1:2,000, BD Biosciences, USA), p-eIF2α (1:500; Abcam, USA), VAMP2 (1:20,000; Abcam, USA), SNAP25 (1:10,000, Abcam, USA), TH (1:1000; Merck-Millipore, USA), p-RIP1 (1:500; Cell Signaling, USA) and p-RIP3 (1:1000; Abcam, USA).

2.10. RNA extraction and real time PCR

Total RNA was isolated from ventral midbrain (containing entire SN), striatum and cortex. After homogenization in PBS, we purified total mRNA using Trizol reagent (Invitrogen, USA) and then cDNA was synthesized from 1 μg of RNA using Reverse Transcription Kit (Applied Biosystems, USA). Quantitative real time PCR was performed in a Stratagene light-cycler system employing the Eva green fluorescent reagent (Biodyne, USA) and the primers listed in Supplementary Table 1. XBP1 mRNA splicing assays in mice (Rodriguez et al., 2012) and rats (Toda et al., 2006) were performed as we previously described.

2.11. Histological analysis

Animals were anesthetized with the mixture of ketamine/xylazine anesthesia (Ketamine: 100 mg/kg, Xylazine: 10 mg/kg) and perfused through the ascending aorta with isotonic saline followed by ice-cold 4% paraformaldehyde in 0.1 M PBS (pH7.4). Brains were removed, post-fixed overnight at 4 °C in the same solution and subsequently placed on 30% sucrose (Merck, USA) at 4 °C for 48 h. Brains were frozen in Optimal Cutting Temperature compound (Tissue Tek, USA), and coronal sections of 25 μm containing the rostral striatum and midbrain were cut on a microtome (Leica, Germany) with a temperature controlled freezing stage (Physi temp Instruments, USA). For immunohistochemical analysis, nigral and striatal tissue sections were quenched with 3% H2O2 at 37 °C for 30 min, blocked at room temperature for 2 h with 0.5% bovine serum albumin and 0.1% triton X-100 and incubated overnight at 4 °C with the primary antibody anti-TH (rabbit 1:1,000; Millipore-Sigma, Germany), then incubated for 2 h at room temperature in a biotinylated goat anti-rabbit antibody (1:500; Vector Laboratories, USA). Sections were then incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, USA) for 30 min at room temperature and developed with 3,3′-diaminobenzidine (Sigma-Aldrich, USA) before mounting on glass slides with Entellan medium (Merck, Germany). Formalin fixed midbrain paraffin sections (5 μm thick) were mounted on superfrost plus tissue slides (Thermo Scientific, USA) and deparaffinized. Subsequently, sections were immersed in 0.3% H2O2 in methanol for 30 min to quench endogenous peroxidase activity. Sections were treated in 10 mM pH6.0 citrate buffer heated in a steamer during 30 min for antigen retrieval and blocked at room temperature for 2 h with 1% goat normal serum and 0.1% triton X-100. Primary antibodies anti-p-eIF2α (rabbit 1:100; Invitrogen, USA) and anti-p-PERK (rabbit 1:500; Santa Cruz Biotechnology, USA) were diluted in blocking solution and applied overnight at 4 °C. Between incubation steps, sections were rinsed with Tris Buffered Saline (TBS, pH7.4). As secondary step, sections were incubated for 2 h at room temperature in a biotinylated goat anti-rabbit antibody (1:500; Vector Laboratories, USA). Sections were then incubated with the avidin-biotin-peroxidase complex (Vector Laboratories, USA) for 1 h at room temperature and developed with 3,3′-diaminobenzidine (Sigma-Aldrich, USA). Images were acquired using an Olympus IX71 microscope.
2.12. Cell counting

To determine the percentage of TH-positive cells loss in the SNpc of injected animals, the number of nigral-dopaminergic cells in the injected and non-injected side was obtained counting in a blinded manner the total number of TH-positive cells obtained by immunohistochemistry in midbrain correlrelative tissue sections every 100 μm containing the entire SNpc as we reported before (Castillo et al., 2015; Valdes et al., 2014). Results were expressed as the percentage of TH-positive neuron loss in the injected side compared to the non-injected side.

2.13. Densitometry analysis of striatal dopaminergic innervation

Striatal dopaminergic innervation was analyzed by measuring the optical density (OD), integrated density of gray pixel values, corrected for non-specific background, of TH immunoreactivity on tissue sections every 100 μm covering the entire striatum. Sections were scanned using an Epson Perfection V600 Photo scanner, and OD of the striatum was analyzed using the ImageJ software (free NIH software: http://rsweb.nih.gov/ij/). Results are expressed as the percentage of TH immunoreactivity loss with respect to the contralateral non-injected hemisphere.

2.14. Statistical analyses

Data are expressed as mean ± SEM. After confirming normal distribution with Skewness/Kurtosis statistic test, Student's t-test was used to analyze differences in histological, behavioral and biochemical analyses. Two-way ANOVA followed by Bonferroni post-test was used in behavioral experiments and body weight. Statistical analyses were performed using GraphPad Prism 5.0 software. Statistical differences were considered significant for values of p < .05.

3. Results

3.1. Activation of PERK/ATF4 signaling in the brain of PD patients and rodent models of the disease

Previous studies have reported the induction of ER stress in PD brain, showing a correlation between the accumulation of Lewy bodies and the activation of PERK at the SNpc (Hoozemans et al., 2007; Selvaraj et al., 2012). To further study the contribution of PERK to PD pathogenesis, we performed a global analysis of the brain of PD patients (Hoozemans et al., 2007; Selvaraj et al., 2012). To further study the contribution of PERK to PD pathogenesis, we performed a global analysis of the brain of PD patients, and prepared them with healthy control subjects (Table 1). We analyzed several brain areas corresponding to the medulla oblongata,pons, midbrain, hippocampus and neocortex. Formalin-fixed paraffin-embedded tissue was stained for phosphorylated PERK (pPERK) and eIF2α (p-eIF2α), followed by quantification of the IHC score (see Methods). A clear increase in the levels of phosphorylated PERK and eIF2α was observed in both PD and incidental cases in all brain areas tested (Fig. 1A, B). In addition, we confirmed the presence of α-Synuclein inclusions in the samples analyzed (Fig. 1C). These results suggest that the activation of PERK signaling in PD cases may occur very early during the disease process based on the positive signals observed in incidental cases.

We next monitored the levels of ER stress in different animal models of PD. Analysis of α-Synuclein transgenic mice expressing the A53T mutation (Gission et al., 2002) at the symptomatic (9–10 months of age) and pre-symptomatic (4 months of age) stages indicated a strong induction of ATF4 using western blot analysis of brain cortex (Fig. 1D). We then analyzed a different model of PD in rats, based on the targeted overexpression of α-Synuclein in the SNpc induced by the unilateral injection of AAV coding for human α-Synuclein (Coune et al., 2011). Three months after AAV delivery, the ventral midbrain, containing the SNpc, of control and injected sides were dissected and the levels of ATF4 target genes were determined using real time PCR. Increased expression of Atf3, Trb3 and Chop mRNA levels was observed in α-Synuclein overexpressing midbrain when compared with the non-injected control sides (Fig. 1E).

We then moved forward and analyzed markers of ATF4 activation in a classical toxicological model of PD. Three months old mice were injected with 8 μg of the neurotoxin 6-hydroxydopamine (6-OHDA) into the right striatum. Animals were euthanized at different time points after injection and the levels of Atf3, Trb3 and Chop mRNA levels measured by real time PCR. A progressive increase in the levels of ATF4-target genes was observed in midbrain tissue starting from 1 week after the injection of 6-OHDA that was sustained for three weeks (Fig. 1F). Consistent with these results, increased levels of phosphorylated PERK (Fig. 1G, Supplementary Fig. S1) and eIF2α (Fig. 1H, Supplementary Fig. S2) were observed in the SNpc of animals injected with 6-OHDA as revealed using IHC. Overall, these experiments indicate that activation of PERK is a salient feature of PD.

3.2. Oral administration of GSK2606414 inhibits PERK signaling at the SNpc

To test the therapeutic potential of GSK2606414 in experimental PD, we first evaluated the bioavailability and the activity of this small molecule in vivo in the target tissue. Mice were treated with GSK2606414 [100 mg/kg/day] through oral administration for 3 weeks. Then, 12 h after the last treatments animals were sacrificed and compound levels detected in serum and brain cortex (see methods). Using this regimen of administration, significant levels of GSK2606414 were detected in both samples, reaching mean concentrations of 4150 ng/mL and 5200 ng/g in serum and tissue, respectively (Fig. 2A). These data confirmed adequate exposure of GSK2606414 in the brain to engage and inhibit PERK, in line with previous studies (Moreno et al., 2015; Radford et al., 2015). We also measured the basal levels of eIF2α phosphorylation and observed a clear reduction in the striatum of mice treated with GSK2606414 using western blot analysis (Fig. 2B).

We then monitored the efficacy of GSK2606414 in inhibiting PERK signaling at the SNpc. To this aim, we performed a pharmacological stimulation of the UPR by the intra-nigral injection of 10 μg of tunicamycin in mice using brain stereotaxis. Animals were pre-treated during 1 week with GSK2606414 [100 mg/kg/day] and then exposed to tunicamycin. Twenty-four hours later, ventral midbrain containing the SNpc was dissected and mRNA levels of Atf3, Trb3 and Chop mRNA were measured by real time PCR. A significant inhibition in the upregulation of Trb3 was observed in GSK2606414 treated animals, whereas Atf3 and Chop only showed a trend of reduction (Fig. 2C). We validated these experiments also in rats by pre-treating animals for 3 days with vehicle or GSK2606414 [100 mg/kg/day] followed by tunicamycin injection. A significant inhibition in the upregulation of Trb3 and Chop was observed after induction of ER stress in this additional animal model (Fig. 2D). To monitor the specificity of the small molecule used, we then determined the levels of XBP1 mRNA splicing, a parallel UPR signaling pathway activated by the stress sensor IRE1α (Walter and Ron, 2011). No effects of GSK2606414 administration were observed on XBP1 mRNA splicing in the same samples (Fig. 3E, F). In addition, no changes in the induction of ATF6 target gene by ER stress were observed when animals were treated with GSK2606414 (Supplementary Fig. S3A, B), indicating that GSK2606414 specifically blocked the activity of PERK without altering other UPR branches.

3.3. PERK inhibition protects dopaminergic neurons against experimental PD

To explore the protective potential of GSK2606414 on dopaminergic neuron survival, we performed unilateral stereotactic injections...
of 8 μg of 6-OHDA into the striatum of mice, followed by the oral administration of GSK2606414 (100 mg/kg/day) or vehicle starting the same day of the neurotoxin injections and continued for a total of three weeks. We analyzed the neurodegenerative process at the SNpc by monitoring nigral-dopaminergic neuron loss using tyrosine hydroxylase (TH) staining by IHC in midbrain tissue as we previously reported (Castillo et al., 2015; Valdes et al., 2014) (Fig. 3A). Importantly, the decrease in TH-positive cells observed in this toxicological model is due to dopaminergic neuron loss and not to the downregulation in the expression of the TH marker as monitored after NeuN or DAT staining (previously described in (Valdes et al., 2014)). We quantified TH-positive cells in serial midbrain tissue sections every 100 μm covering the entire SNpc (Supplementary Fig. S4). Using this method, we observed a 49% of neuronal loss in vehicle treated animals, whereas GSK2606414 provided strong neuroprotection reflected on a 29% reduction in the content of TH-positive cells (Fig. 3B, left panel), indicating an efficacy of protection close to the 45% (Fig. 3B, right panel). We also detected the protection induced by GSK2606414 by measuring TH protein levels in the SNpc using western blot analysis (Supplementary Fig. S5). As expected, a similar degree of striatal denervation induced by 6-OHDA (loss of TH-positive staining) was observed in vehicle and GSK2606414 treated animals, indicating that the neurotoxin was equally effective in inducing damage at the striatum in both groups (Fig. 3C, Fig. 4H, Supplementary Fig. S6). Taken together, these results indicate that the pharmacological targeting of PERK protects dopaminergic neurons against a parkinsonian-inducing neurotoxin.

3.4. GSK2606414 administration improves motor performance, and recovers dopamine content and synaptic protein expression on a model of PD

To determine if the neuroprotective effect of GSK2606414 treatment translate into improved motor control, we performed several behavioral tests in animals injected with 6-OHDA, including cylinder test, hanging wire test and the rotarod. Mice were monitored in the cylinder test to measure forepaw akinesia over time after 6-OHDA injection, and baseline recorded one week prior to the experiment. Forepaw akinesia was strongly induced by the 6-OHDA hemilateral lesion only in vehicle treated animals, while GSK2606414 treated animals show a slight increase in forepaw akinesia that was not significant at any time point analyzed (Fig. 4A). Then, we performed the hanging wire test, which measures coordination and muscle strength of animals. Remarkably, the drop observed in the performance of animals in this test 14 days post 6-OHDA injection was completely inhibited in the GSK2606414 treated group (Fig. 4B, Supplementary Movies 1 and 2). Similarly, using rotarod assay, GSK2606414 administration reduced the decay in performance observed 21 days after 6-OHDA injection (Fig. 4C). Taken together, these results indicate that the protection of dopaminergic neurons observed at the histological level induced by GSK2606414 treatment translated into a significant attenuation in the motor and coordination defects.

To obtain insights about the physiological and functional state of dopaminergic neurons after GSK2606414 administration, we monitored the levels of dopamine and its metabolite DOPAC using Ultra Performance Liquid Chromatography–Tandem Mass Spectrometry (UPLC-MSMS). 6-OHDA triggered a reduction near to 60% in the levels of DOPAC and dopamine after 21 days of injection (Fig. 4D). Remarkably, a complete recovery in the levels of DOPAC and dopamine were observed in in GSK2606414 treated animals (Fig. 4D).

One of the pathological effects of chronic PERK signaling is the repression in the synthesis of certain synaptic proteínes (Smith and Mallucci, 2016). Importantly, changes in synaptic protein content have been reported in PD models including the administration of 6-OHDA, associated with alterations in the expression of SNAP25 and VAMP2 (Xiong et al., 2014). We measured protein levels of these two synaptic markers in dissected striatum using western blot analysis (Supplementary Fig. S5). As expected, a similar degree of striatal denervation induced by 6-OHDA (loss of TH-positive staining) was observed in vehicle and GSK2606414 treated animals, indicating that the neurotoxin was equally effective in inducing damage at the striatum in both groups (Fig. 3C, Fig. 4H, Supplementary Fig. S6). Taken together, these results indicate that the pharmacological targeting of PERK protects dopaminergic neurons against a parkinsonian-inducing neurotoxin.

### 3.5. GSK2606414 induces slight body weight loss and pancreatic toxicity in a model of PD

Previous studies indicated adverse effects of GSK2606414 administration due to pancreas toxicity (Atkins et al., 2013; Halliday et al.,
However, animals were able to undergo treatments for months leading to important neuroprotective effects in models of PrDs and frontotemporal dementia (Moreno et al., 2013; Radford et al., 2015). In our experimental setting, oral administration of GSK2606414 [100 mg/kg/day] for a period of for 21 days after 6-OHDA injection led to a decrease in body weight of around 19% (Fig. 5A). This drop in body weight was accompanied by transient and small fluctuations in glucose levels that were not significant at 21 days post 6-OHDA injection (Fig. 5B). Histological analysis of the pancreas using hematoxylin eosin staining indicated that administration of GSK2606414 for 21 days led to signs of tissue damage with disintegrated pancreatic acini and lower content of islets when compared with vehicle-treated animals (Fig. 5C). These experiments indicate that although GSK2606414 treatment has strong neuroprotective effects on our PD model, it results in slight undesired side effects possibly related to pancreatic dysfunction.

4. Discussion

Parkinson’s disease is an invalidating condition with no available
Although several pathological mechanisms have been proposed to explain the loss of dopaminergic neurons in PD, several studies indicate that proteostasis disruption at the level of the ER is a salient and transversal feature of the disease. Chronic ER stress may result in deleterious events that promote dopaminergic neuron dysfunction and death. The current view of the involvement of the UPR in neurodegenerative diseases is highly complex due to the divergent roles of specific components of UPR in distinct diseases (Hetz and Saxena, 2017; Hoozemans et al., 2007; Smith and Mallucci, 2016). Studies in mouse models of PD suggested that activation of the ATF6 and IRE1α/XBP1 signaling branches are neuroprotective, involving an increased buffering capacity, reducing ER stress levels (Credle et al., 2015; Egawa et al., 2011; Sado et al., 2009; Valdes et al., 2014). Moreover, under resting conditions these two signaling pathways have been proposed to balance proteostasis to sustain dopaminergic neuron physiology (Egawa et al., 2011; Valdes et al., 2014). Although ATF4/CHOP expression has a relevant role in inducing neuronal loss in toxin-based models of PD (Gully et al., 2016; Silva et al., 2005), the therapeutic potential of targeting PERK signaling as an intervention strategy has not been explored. Recent advances in the field led to the identification of several small molecules to inhibit or activate specific UPR signaling branches.

In the current study, we investigated the potential of inhibiting the enzymatic activity of PERK to improve the function and survival of dopaminergic neurons. We validated the presence of ER stress and signs of PERK/ATF4 activation in human brain tissue derived from PD patients, in addition to incidental cases of PD. Moreover, we confirmed that the pathway is engaged in various rodent models of PD as an early response even before behavioral alterations are observed. Motivated by these observations we then determined the therapeutic potential of inhibiting PERK signaling with the small molecule GSK2606414 in an animal model of PD.

The oral administration of GSK2606414 is effective in preventing neurodegeneration and synaptic dysfunction in models of PrPs and tauopathies (Moreno et al., 2013; Radford et al., 2015). GSK2606414 administration also reduces tissue damage and cognitive impairment in a model of brain injury induced by hemorrhage (Yan et al., 2017).
Our current study is the first demonstrating that pharmacological inhibition of PERK has neuroprotective potential at the SNpc. A previous report used a generic approach to target the ISR in two models of α-Synuclein-mediated using salubrinal (Colla et al., 2012a), a small molecule that inhibits the two eIF2α phosphatase complexes (Boyce et al., 2005). That study demonstrated neuroprotection at the behavioral level, improving motor performance, however, no protection by salubrinal administration was observed at the level of nigral-dopaminergic neurons (Colla et al., 2012a). Importantly, alternative substrates for PERK kinase have been identified including NRF2 (Cullinan et al., 2003), which controls the antioxidant response (Cullinan and Diehl, 2004) and is implicated in neurodegenerative diseases (Johnson and Johnson, 2015). Due to the side effects of GSK2606414 to pancreatic function, the use of this drug for the treatment of PD patients has low translational potential (Mercado and Hetz, 2017). However, as proof-of-concept the use of the PERK inhibitor allowed us to propose that new safe molecules to target the pathway may have the ability to attenuate neurodegeneration in PD. One recent study questioned the specificity of GSK2606414 and suggested a higher affinity for RIP1 kinase (Rojas-Rivera et al., 2017), a central component of the necroptosis machinery. However, that study only used artificial in vitro systems to characterize this activity and our data confirmed the direct effects of GSK2606414 on PERK signaling. In addition, we did not detect signs of RIP1 or RIP3 kinase activation in our experimental model (Supplementary Fig. S7). Another small molecule known as ISRIB inhibits the consequences of eIF2α phosphorylation by binding to the eIF2B complex and preserving global translational capacity (Sekine et al., 2015; Sidrauski et al., 2015). ISRIB improves synaptic function at basal levels (Sidrauski et al., 2013), and provided neuroprotection in models of PDs without signs of pancreatic toxicity (Halliday et al., 2015). However, ISRIB has poor solubility and lower relative activity compared with PERK in inhibiting protein translation (Sekine et al., 2015; Sidrauski et al., 2015). Interestingly, a recent report screened for molecules that mimic the cellular effects of ISRIB using a library of FDA-approved drugs (Halliday et al., 2017). This study identified dibenzoylmethane and trazodone as strong candidates to target the ISR, having significant therapeutic effects in models of neurodegeneration (Halliday et al., 2017). We are currently testing the efficacy of these drugs in PD models.

Overall, it is predicted that the prolonged administration of compounds that target PERK will have deleterious effects to the function of highly secretory cells like pancreatic beta cells, exocrine pancreas and other tissues that require PERK signaling to sustain their normal function (Dufey et al., 2014). Thus, in the scenario of treating a PD patient (10–15 years of treatment), inhibiting the PERK pathway using systemic administration of small molecules carries a high risk of adverse side effects. In addition, studies using genetic ablation of PERK in the brain have shown important defects in the basal activity of cortical neurons, leading to behavioral alterations (Costa-Mattioli et al., 2009; Ounallah-Saad et al., 2014; Trinh et al., 2012a; Trinh and Klann, 2013). Furthermore, reduction of eIF2α phosphorylation enhances long-term memory formation (Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007; Ounallah-Saad et al., 2014; Zhu et al., 2011). The role of the UPR downstream target ATF4 in neuronal physiology is controversial given both negative and positive results on behavioral tasks (Bartsch et al., 1995; Costa-Mattioli et al., 2005; Costa-Mattioli et al., 2007; Chen et al., 2003; Pasini et al., 2015; Trinh et al., 2012b). GSK2606414 was shown to alter the basal function of certain neuronal circuits, where it can enhance taste-dependent learning and plasticity (Ounallah-Saad et al., 2014), whereas it impairs working memory (Zhu et al., 2016). Thus, strategies for the local manipulation of PERK in the SNpc should be considered in the future using gene therapy to downregulate PERK and thus avoid the systemic effects generated by PERK inhibition. In support of this idea, delivery of AAVs to express ATF4 into the SNpc triggers spontaneous dopaminergic neuron apoptosis (Gully et al., 2016). Our study suggests that drugs to alleviate ER stress levels may have important beneficial effects in PD. Two major molecular targets of

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**Fig. 3.** GSK2606414 treatment protects dopaminergic neurons against 6-OHDA-induced neurotoxicity. (A) Wild-type mice were hemilaterally injected with 6-OHDA into the right striatum, and from the same day were treated with vehicle or GSK2606414 [100 mg/kg/day]. After 3 weeks, dopaminergic neurons were visualized in midbrain tissue sections by anti-TH immunostaining. Scale bar: 500 μm. (B) Anti-TH immunohistochemistry was performed in midbrain tissue sections every 100 μm and nigral dopaminergic neurons were quantified and expressed as a percentage of neuron loss compared to the non-injected (control) side (left panel). In addition to calculate normalized neuronal damage using vehicle treated animals as 100% (right panel). (C) Anti-TH immunohistochemistry analysis was performed in striatal sections to quantify 6-OHDA-induced denervation in both injected and non-injected sides. Scale bar: 1 mm. (D) The integrated density of pixel intensity was calculated from images of anti-TH immunohistochemistry covering the entire striatum and expressed as the percentage of loss compared with the non-injected (control) sides. For quantifications, mean and SEM are presented (n = 12 for vehicle; n = 14 for GSK2606414). For statistical analysis, mean and SEM are presented followed by Student’s t-test. **: p < .01.

However, the ability of GSK2606414 to target the SNpc has not been tested before. Our results demonstrate that GSK2606414 is active in the brain and specifically engages the target in a model of chronic ER stress. For proof-of-concept, we used a rapidly evolving model of PD in mice based on the injection of a neurotoxin that selectively damages dopaminergic neurons. We found that the oral administration of GSK2606414 augmented the survival of dopaminergic neurons at the SNpc, associated with increased dopamine levels and motor functions. At the molecular level, PERK inhibition may not only reduce deleterious proapoptotic signals downstream of ATF4, but may also improve dopaminergic neuron function by restoring the levels of synaptic proteins. These effects contrast with the known role of eIF2α phosphorylation as a general stress mitigation output of ISR.
α-Synuclein that perturbed ER function involve the direct inhibition of ER to Golgi trafficking (Cooper et al., 2006; Gitler et al., 2008) and also ATF6 inhibition (Credle et al., 2015), resulting in deleterious levels of stress. Gene therapy is emerging as a feasible strategy to specifically target the SNpc and deliver therapeutic genes (Castillo, Mercado, and Hetz, 2015; Coune et al., 2012). We speculate that a more direct way to reduce ER stress and improve the functionality and survival of dopaminergic neurons will be the delivery of active XBP1, ATF6, or ER chaperones to the SNpc. For example, the enforced expression of XBP1s into the SNpc using recombinant viruses has been shown to protect dopaminergic neurons against PD-inducing neurotoxins (Sado et al., 2009; Valdes et al., 2014). Similarly, the local delivery of BiP into the SNpc has a strong neuroprotective effect on a model of α-Synuclein-induced degeneration (Gorbatsyuk et al., 2012).

The therapeutic potential of the UPR to treat PD may impact other aspects of the disease that are beyond synaptic protein expression and ER stress buffering. In this line, we have described new biological functions of XBP1, where it can enhance synaptic plasticity by controlling the expression of BDNF (Martinez et al., 2016), in addition to improving axonal regeneration (Onate et al., 2016). AAV-XBP1s injections can also improve motor function after axonal damage (Valenzuela et al., 2012), in addition to reduce abnormal protein aggregation in models of Huntington’s disease (Zuleta et al., 2012). Additional studies have also demonstrated that strategies to alleviate ER stress using gene

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Fig. 4. GSK2606414 treatment improves motor performance and dopaminergic neuron function. Animals were injected with 6-OHDA in the right striatum and from the same day treated with GSK2606414 [100 mg/kg/day] or vehicle. (A) Spontaneous forepaw use during exploratory behavior was assessed over time by the cylinder test. Touches on a cylinder wall were scored for each forepaw. Results were plotted as a percentage of touches with left forepaw (contralateral to injection side) in relation to the total of number touches with both forepaws. (B) Motor performance was quantified using a hanging test as indicated in material and methods to calculate a score of the performance. (C) Motor coordination was assessed by rotarod test. The rotarod was set with a start speed of 4 rpm, acceleration rate 20 rpm/min, and the time of latency before fall was recorded. The mean on five trials for each animal is presented as a percentage from the base line performance. In all tests mean and SEM are presented (n = 9 for vehicle; n = 10 for GSK2606414). Statistical analysis was performed by two ways ANOVA followed by Bonferroni post-test: *: p < .05 and **: p < .01 and ***: p < .001. (D) Biogenic monoamines including DOPAC and dopamine were measured by UPLC-MSMS in the 6-OHDA injected and non-injected (control) striatum of vehicle and GSK2606414 treated animals. Results are presented as percentage of the corresponding non-injected sides in means and SEM. Statistical analysis were performed by two ways ANOVA followed by Bonferroni post-test: **: p < .01 and ***: p < .001. (n = 4 per vehicle and n = 7 per GSK2606414). (E) Levels of the synaptic proteins VAMP2, SNAP25 and TH were determined by Western blot in striatal tissue dissected from vehicle and GSK2606414 [100 mg/kg/day] treated animals from the 6-OHDA lesioned and the non-injected (control) sides. β-actin was measured as loading control. (F) Levels VAMP2 protein were quantified relative to β-actin (n = 4 per group). (G) Levels SNAP25 were quantified relative to β-actin (n = 4 per group). (H) Levels TH were quantified relative to β-actin (n = 4 per group). For statistical analysis, mean and SEM are presented followed by Student's t-test. *: p < .05.
Fig. 5. GSK2606414 treatment induces body weight loss and pancreatic dysfunction. Wild-type mice were injected with 8 μg of 6-OHDA in the right striatum and treated with vehicle or GSK2606414 [100 mg/kg/day] for 21 days. (A) Body weight was measured over time during treatment and presented as mean and SEM per group. Statistical analysis was performed by two ways ANOVA followed by Bonferroni post-test: ***: p < .001. (B) Glucose in blood was determined and expressed as [mg/dL]. For statistical analysis, mean and SEM are presented followed by Student’s t-test. *: p < .05. (C) Upper panels: representative images of hematoxylin and eosin-stained pancreas sections of vehicle and GSK2606414 [100 mg/kg/day] treated animals. Arrowheads indicate pancreatic islets. Scale bar: 100 μm. Lower panels: show magnifications of the insets indicated by the cations of the insets indicated by the 

therapy are neuroprotective in various neurodegenerative diseases (Valenzuela et al., 2016a). GSK2606414 was also shown to attenuate ER stress-induced brain inflammation, impacting cytokine production and glial activation (Guthrie et al., 2016), suggesting additional beneficial impacts of targeting the UPR in brain diseases. More efforts are needed to define the significance of the UPR as a target to treat PD. In summary, our results demonstrate the significance of PERK signaling to dopaminergic neuron function and survival on a model of PD. This study may also contribute to understanding the differential neuronal vulnerability observed in PD patients.

5. Conclusions

In this report we provide evidence indicating that the pharmacological inhibition of PERK using the oral delivery of GSK2606414 results in strong neuroprotection in a model of PD. PERK inhibition also increases dopamine levels and motor performance possibly associated with an augmented levels of key synaptic proteins. Additional studies using alternative approaches to locally target this pathway at the SNpc are needed to develop potential therapies for PD.

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Conflict of interest


Author contributions

G.M. and C.H designed the study. G.M. and V.C. participated in experimental design, performed experiments and analyzed the data. P.S. and N.L. performed experiments. J.M.A. provided GSK2606414 compound expertise and contributed to critical discussions of experimental design and data analyses. S.P.S contribute with the AAV vectors to over-express α-Synuclein. J.J.M.H. performed human tissue samples studies. G.M., V.C. and C.H. wrote or contributed to writing the manuscript. All authors read and approved the final version of the manuscript.

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