ORIGINAL ARTICLE

GPI-1046 Increases Presenilin-1 Expression and Restores NMDA Channel Activity

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ABSTRACT: ***Background:*** Previously we showed that 6-hydroxydopamine lesions of the substantia nigra eliminate corticostriatal LTP and that the neuroimmunolophilin ligand (NIL), GPI-1046, restores LTP. ***Methods:*** We used cDNA microarrays to determine what mRNAs may be over- or under-expressed in response to lesioning and/or GPI-1046 treatment. Patch clamp recordings were performed to investigate changes in NMDA channel function before and after treatments. ***Results:*** We found that 51 gene products were differentially expressed. Among these we found that GPI-1046 treatment up-regulated presenilin-1 (PS-1) mRNA abundance. This finding was confirmed using QPCR. PS-1 protein was also shown to be over-expressed in the striatum of lesioned/GPI-1046-treated rats. As PS-1 has been implicated in controlling NMDA-receptor function and LTP is reduced by lesioning we assayed NMDA mediated synaptic activity in striatal brain slices. The lesion-induced reduction of dopaminergic innervation was accompanied by the near complete loss of NDMA receptor-mediated synaptic transmission between the cortex and striatum. GPI-1046 treatment of the lesioned rats restored NMDA-mediated synaptic transmission but not the dopaminergic innervation. Restoration of NDMA channel function was apparently specific as the sodium channel current density was also reduced due to lesioning but GPI-1046 did not reverse this effect. We also found that restoration of NMDA receptor function was also not associated with either an increase in NMDA receptor mRNA or protein expression. ***Conclusion:*** As it has been previously shown that PS-1 is critical for normal NMDA receptor function, our data suggest that the improvement of excitatory neurotransmission occurs through the GPI-1046-induced up-regulation of PS-1.

RÉSUMÉ: Le GPI-1046 augmente l'expression de la préséniline 1 et rétablit l'activité des canaux NMDA. ***Contexte :*** Nous avons démontré antérieurement que les lésions de la substance noire induites par la 6-hydroxydopamine éliminent la LTP (potentialisation à long terme) corticostriée et que le ligand de la neuroimmunolophiline (NIL), GP-1046, rétablit la LTP. ***Méthodes :*** Nous avons utilisé des puces à ADNc pour déterminer quels ARN peuvent être sur ou sous-exprimés en réponse à une lésion et/ou au traitement par le GPI-1046. Nous avons effectué des enregistrements patch-clamp pour évaluer les changements de la fonction des canaux NMDA avant et après traitement. ***Résultats :*** Nous avons observé l'expression différentielle de 51 gènes. Parmi ceux-ci, nous avons constaté que le traitement par le GPI-1046 régulait à la hausse la quantité d'ARNm de la préséniline 1 (PS1). Nous avons confirmé cette observation par Q-PCR. Nous avons également démontré que la PS1 était sur-exprimée dans le striatum de rats traités par le GPI-1046. Étant donné que la PS1 a été impliquée dans le contrôle de la fonction des récepteurs NMDA et que la LTP est diminuée par ces lésions, nous avons mesuré l'activité synaptique médiée par le NMDA dans des coupes de striatum. La diminution de l'innervation dopaminergique consécutive à la lésion était accompagnée par la perte presque complète de la transmission synaptique médiée par les récepteurs NDMA entre le cortex et le striatum. Le traitement par le GPI-1046 de rats ayant subi une lésion a restauré la transmission synaptique médiée par le NMDA, mais non l'innervation dopaminergique. La restauration de la fonction des canaux NDMA était, semble-t-il, spécifique étant donné que la densité du courant des canaux sodiques était également diminuée par la lésion mais le GPI-1046 ne corrigeait pas cet effet. Nous avons également constaté que la restauration de la fonction du récepteur NMDA n'était pas associée à une augmentation de l'ARNm du récepteur NMDA ou de l'expression de la protéine. ***Conclusion :*** Nos données suggèrent que l'amélioration de la neurotransmission excitatrice se produit au moyen de la régulation à la hausse de la PS1 induite par le GPI-1046, conformément à ce qui a été démontré antérieurement, à l'effet que la PS1 est critique pour la fonction normale du récepteur NMDA.

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Neuroimmunophilin ligands (NILs) have shown efficacy in a number of disease models reversing the effects of 6-hyroxydopamine lesions to the substantia nigra (a model of Parkinson's disease[1-3], ameliorating memory loss in Alzheimer's disease models[4] and preventing nerve fibre loss as a result of trauma (surgical or otherwise)[5-15]. NILs are derived from the natural product and immunosuppressant, FK506 (clinical name: tacrolimus), first isolated from Streptomyces tsukubaensis, a soil fungus[16]. However, the neurotrophic/regenerative activity does not appear to be related to cellular mechanisms involved in immune system function. This was recognised early on when NILs such as GPI-1046[17] were shown to enhance neuronal sprouting and survival but did not possess any immuno-suppressant activity. So, although NILs bind to a large class of proteins called FK506-binding proteins (FKBPs) that interact with and inhibit calcineurin, a serine/threonine protein phosphatase involved in regulating immune function[18], their action in neurons does not involve the inhibition of calcineurin[17]. To date, the mode of action of these molecules has not been fully established. Indeed, the efficacy of these ligands in a range of disease models suggests that the action may be state-dependent (injured or not) as well as cell-type-dependent (for review see [19]). In our previous work[3] we showed that after 6-OHDA lesioning of the substantia nigra pars compacta (SNc), GPI-1046 restores striatal tyrosine hydroxylase (TH) immunoreactivity and cortically induced striatal long-term potentiation (LTP). The recovery was not correlated with any cytoprotective effects in the SNc. Thus the restoration of near-normal function was associated with the GPI-1046-induced re-innervation of the striatum by SNc neurons that had survived the initial insult. We also found in sham-lesioned animals and in lesioned rats after treatment, LTP was completely abolished by either dopamine or NMDA receptor antagonists. Thus, it seemed clear that the restoration of dopaminergic function was necessary for LTP. As the loss of dopaminergic function is likely to have an effect on gene expression and NILs are known to bind to proteins involved in transcriptional regulation[19], we were interested in discovering how mRNA abundance may be altered by the lesioning process and/or GPI-1046 treatment. Furthermore the loss of dopamine likely induces a loss of NMDA receptor function and we hypothesised that GPI-1046 treatment may induce its restoration through an effect on re-innervation. In order to investigate this hypothesis we used cDNA microarays, QPCR, immuno-histochemistry and patch clamp recording techniques to study gene/protein expression and their relationship to the functional activity of striatal neurons. We found that the restoration of function was correlated with the GPI-1046 induced up-regulation of presenilin-1 (PS-1).

Materials and Methods

The Parkinson's disease (PD) animal model and treatment with GPI-1046

All animal experiments were conducted in accordance with Canadian Council on Animal Care guidelines, and with the approval of the Carleton University Animal Care Committee. 6-OHDA was injected into the right or left substantia nigra to produce the PD model. Adult male Long-Evans Hooded or Sprague Dawley rats, weighing 200-225 g, were anaesthetized and the tip of a microinjection cannula was placed in the substantia nigra (co-ordinates relative to Bregma: P -5.2 mm, L or R 2.0 mm and H -8.0 mm). 6-OHDA, dissolved in 0.4% ascorbic acid to prevent oxidation, was injected (2 µg per rat) through the cannula in a volume of 2 µl within 3 minutes. Control animals were injected with vehicle (0.4% ascorbic acid) in the same manner. Two to three weeks after surgery, rotational behaviour induced by amphetamine (3 mg/kg, i.p.) was used to evaluate the effectiveness of 6-OHDA injections. Rats showing strong ipsilateral rotation were regarded as successful subjects and divided into two groups. One received injections of GPI-1046 (10 mg/kg, s.c., daily) for one week. GPI-1046 was dissolved in 100% ethanol and then diluted with intralipid solution. The other (control) group received injections of vehicle only (intralipid and alcohol).

cDNA microarray preparation. All cDNA microarrays (15k Mouse microarrays) were obtained from the University Health Network (UHN) Microarray Centre in Toronto, ON, Canada. Each slide was hybridized with labeled cDNA from either sham-lesioned, lesioned, or GPI-1046-treated lesioned rats. Reverse transcription to directly label cDNA with either Cyanine 3 (Cy3) or Cyanine 5 (Cy5) was performed in a 40 µl reaction tube containing 8 μl 5x First Strand reaction buffer, 1.5 μl Anchored dT primer (100 picomoles/μl), 3.0 μl deoxynucleotide triphosphates (dNTPs) (6.67 mM each dATP, dGTP, dTTP), 1.0 μl dCTP (2mM), 1.0 μl Cy3 or Cy5 (1mM), 4 μl DTT (0.1M), 10 μg total RNA and RNAse-free water to 37 μl. The enzyme-free reaction mixture was incubated at 65ºC for five minutes to denature the RNA and then cooled to 4ºC. Reverse transcriptase enzyme (2 μl) was added along with RNAse inhibitor (1μl). This mixture was incubated at 42ºC for 2.5 hours.

The reactions were placed on ice and 7 μl 0.5M NaOH/50mM EDTA added to each tube and incubated at 65ºC for ten minutes, placed on ice, and 10 μl 1M Tris-HCl (pH 7.5) added. The two tubes containing Cy3 mixture and Cy5 mixture were combined with 16 μl 10 mM Tris (pH 8.0)/1 mM EDTA. A Microcon YM-30 filter device was employed to concentrate the 130 μl mixture to ~5 μl by adding 100 μl 1X TE (pH 8.0) to the Microcon reservoir and centrifuging at 14,000 x g for three minutes. The RT reaction was added to the reservoir and centrifuged for another ten minutes. The tube was removed from the reservoir and replaced with a clean one. Next, 5-μl 1X TE buffer (10 mM Tris-HCl, pH 8.0/ 1 mM EDTA) was added to the reservoir and the sides of the reservoir were tapped gently. The reservoir was placed upside down in the clean microfuge tube and centrifuged for two minutes. The concentrated cDNA samples were kept on ice until hybridization. The hybridization solution was prepared by combining 5 μl 10 mg/ml) yeast transfer RNA and 5-μl calf thymus DNA 5 mg/ml with 100 μl DIG Easy Hyb solution. This solution was heated for two minutes at 65ºC; 50 μl was added to the concentrated cDNA (~5 μl) solution and held for two minutes at 65ºC. The heated mixture was pipetted onto a preheated microarray slide and incubated overnight (8-18 h, 37ºC) in humidified and light-free atmosphere.

The next day the slides were rinsed in 1X saline-sodium citrate buffer (SSC) and washed three times for ten minutes at 50ºC in 1X SSC, containing 0.1% sodium dodecyl sulfate (SDS). After washing, the slides were given a final rinse in 1X SSC and centrifuged until dry. A GenePix 4000B Microarray Scanner employing GenePix Pro 4.0 array acquisition and analysis

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software was used. Ratios between the Cy3 and Cy5 signals were used to assay for gene expression differences between tissue samples. The design of the experiments was done according to the 2X2 factorial design as described in[20]. Analysis for significant expression differences was done using GeneSpring Software.

RNA extraction/isolation and QPCR. Total cellular RNA from striatal tissue was isolated using Trizol reagent according to the manufacturer's protocol. The isolated RNA pellet was dissolved in 20 µl RNAse-free water. A 1 µl sample of this was added to 99 µl (1X) tris-ethylenediaminetetraacetic acid (TE) buffer analyzed for nucleic acid concentration and purity using a Bio-Rad Smart Spec 3000 spectrophotometer. Acceptable RNA samples with a 260/280 ratio between l.8 and 2.0 were used in all experiments. RNA was stored in 50% ethanol solution at -80°C. The expression of PS-1 and NR-1 mRNA was assayed in a QPCR reaction that was run in a 30-µl volume containing 1 U of TAQ enzyme using a Stratagene Mx4000 real-time PCR thermocycler using SYBR-green. Primer sequences were, forward: PS-1TCC GTG TCA ATC AAG CAG TGC; reverse: CAG CCA CCC GTC ACT TTA ATT C. NR-1: ATG TTC CGC GAG GCA GTA AAC C reverse TCT GTA TGG AGT TGG GXT TGT G. Both primer sets were verified as being greater than 90% efficient by plotting the cycle threshold (done in duplicate) against a known quantity of reversed transcribed total RNA (see Figures 1 and 6). In an assay the cycle threshold for each assay was performed in duplicate for each animal and was normalized against a reference gene, synaptophysin (SYN)[21]. The difference between PS-1 or NR1 cycle thresholds and the SYN cycle threshold (normalized cycle threshold Ctn) was used for statistical analysis, as described[21].

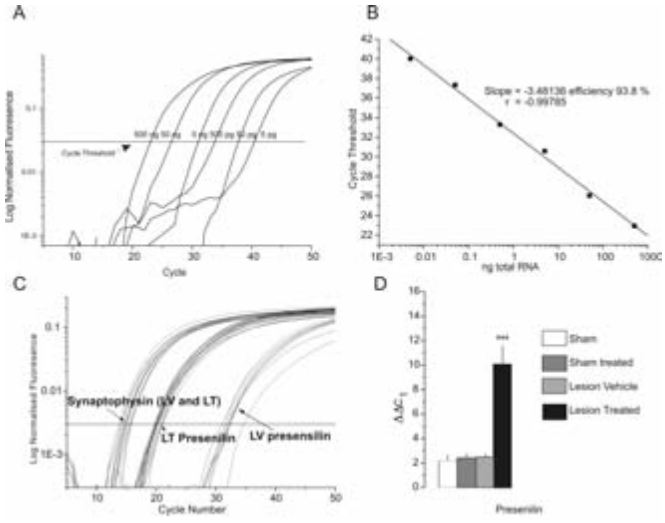

Figure 1: *NIL treatment causes an up-regulation in Presenilin-1 mRNA expression. Panel A shows the results of a standard curve experiment where a QPCR run was set up by varying quantities of reverse-transcribed total cellular RNA. The cycle threshold is set at 20 X above background. The results of this experiment show that an excellent relationship between total cellular RNA and cycle threshold was obtained. In panel B the slope of the relationship indicated that primer efficiency was about 94%. In panel C we show a representative experiment in which PS-1 is up-regulated in lesioned tissue. In panel D we show the quantification of these experiments; note that GPI-1046 up-regulated PS-1 only if the tissue was lesioned.*

PS-1, Tyrosine hydoxylase (TH) and NMDA receptor-1 (NR-1) immunohistochemistry

Rats were deeply anaesthetized and perfused with Lana's fixative (4% PAF and 1.2% picric acid in PBS) or modified Zamboni and de Martino fixative (4% PFA and 0.2% picric acid in PB). The brains were then removed and further fixed in 4% paraformaldehyde overnight, then transferred to a 30% sucrose solution for a minimum of 48 hours. Frozen slices of 40-µm thickness were cut from brain areas containing striatum or substantia nigra. For immunocytochemistry the slices were washed three times with PBS containing 0.2% Triton-X 100, followed by incubation with universal blocking solution (DAKO Diagnostics Canada Inc., Mississauga) for 30 minutes. Slices were then incubated with either: 1) anti-PS-1 antibody (Santa Cruz, Santa Cruz CA 1:250 dilution); 2) tyrosine hydroxylase anti-TH antibody (1:2500 dilution, Pel Freeze Inc., Rogers, Arkansas); or 3) the NR-1 antibody (1:500, RBI, Mississauga, ON) at 4°C overnight, washed, then incubated with secondary antibody labelled with Alexa 488 (PS-1, 1:500) or CY3 (TH and NR-1, 1:400 dilution; Jackson Labs) for 60 minutes. Following removal of secondary antibody solution, slices were washed 3 times with PBS and transferred to glass slides for analysis. Counterstaining of cell nuclei for the PS-1 immunocyto-chemistry was done using DAPI.

Confocal images were taken with an Olympus IX51 microscope equipped with a Perkin Elmer Spinning disk confocal attachment. All exposure times for each photo were identical for each condition (sham, sham-treated lesion vehicle and lesion-treated). In order to quantify the intensity of the fluorescent signals the number of pixels that were at least 2X the background were counted in regions of interest (ROI) in the striatum near (just below the white matter) locations where electrophysiological recordings were made. For each animal and each condition, three ROIs were assayed. The median pixel intensity determined from an intensity/pixel frequency histogram was used as background. The percentage of pixels 2X greater than threshold was counted and this value was then normalised by dividing by the total number of pixels in the ROI. Data shown in Figure 2 are normalized to Sham/vehicle group. Contrast enhancement used for photos shown in Figure 2 was identical. Figure 7 images were taken on a wide field epifluoresence microscope.

Electrophysiological recordings from brain slices

The electrophysiological portion of the study was undertaken two to five weeks after treatment with vehicle or GPI-1046. By the time rats were used for the electrophysiological portion of the study they weighed between 400 and 600 grams and were between three and five months of age. Rats were anaesthetized and their brains removed as previously described[22]. Brain slices of 300-400 µm thickness containing the striatum were prepared using a vibratome in ice-cold ACSF. After slicing, the sections were incubated at 37° for 30 minutes in gassed ACSF (and then subsequently maintained at room temperature).

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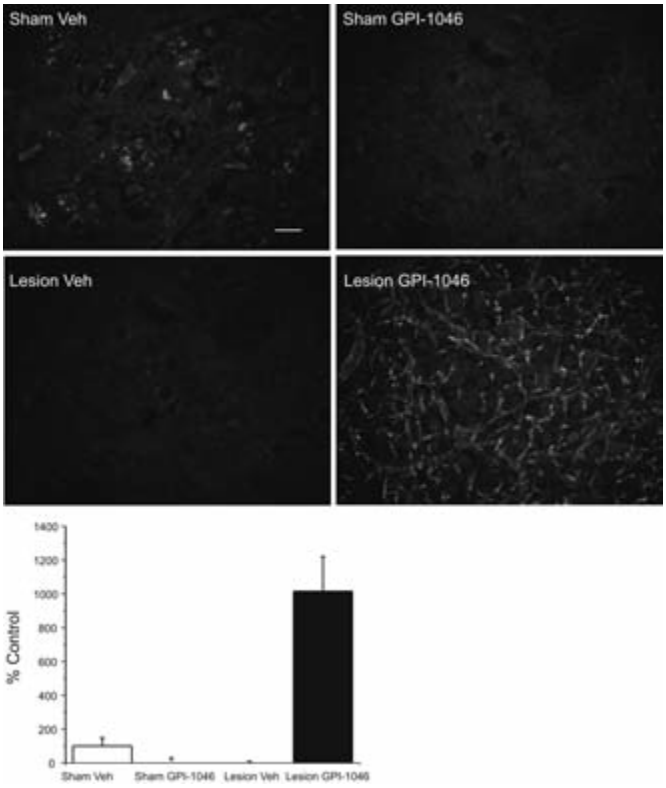

Figure 2: *GPI-1046 treatment up-regulates PS-1 expression in the lesioned/treated rats. Confocal pictures (40 X objective) show PS-1 immunoreactivity was detected in the cell bodies and processes of striatal cells of Sham/Vehicle (Veh) but little is detected in sham GPI-1046 treated or Lesion/Veh groups. By contrast, much greater expression was found in the striata of rats that had been lesioned and treated with GPI-1046. Expression of PS-1 in the ipsilateral striatum was greater than in striatum contralateral to the lesion (not shown). Scale bar is 10 μm.*

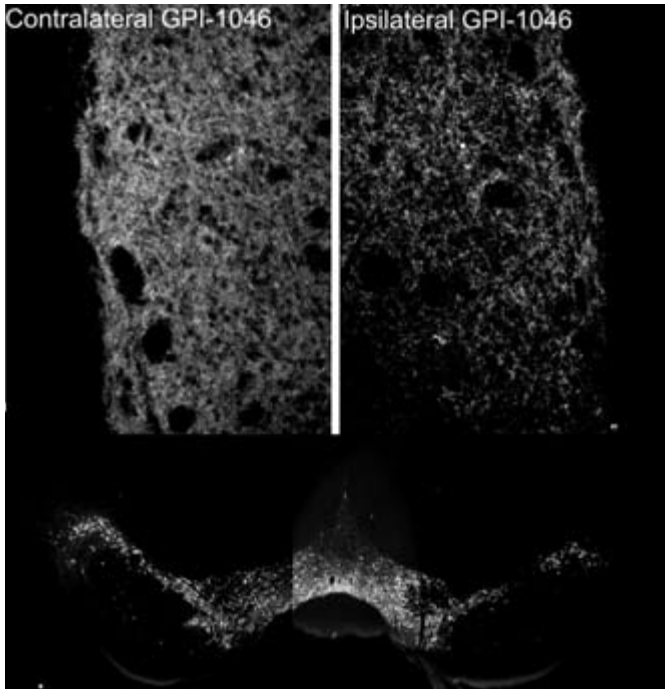

Figure 3: *GPI-1046 has no effect on tyrosine hydroxylase (TH) activity staining after 6-OH dopamine lesion. In Long Evans hooded rats, NILS treatment failed to induce the recovery of TH, unlike that seen in the Sprague-Dawley strain (cf. [2,3]). Top Left shows TH immunoreactivity of the contralateral striatum and the corresponding non-lesioned substantia nigra compacta (SNc). Panel at Top Right shows diminished TH immunoreactivity, even though the animal had been treated with GPI-1046; the corresponding lesioned SNc is shown below.*

Recordings were made from striatal cells both ipsilateral and contralateral to the lesioned SNc injection site in rats that had either been treated with GPI-1046 or sham-injected with vehicle. Slices were placed in a recording chamber and continuously perfused with gassed ACSF (Mg^{2+}-free) at room temperature. The perfusion rate was adjusted to 2 ml/min. Recordings were made from the striatum near the overlying white matter (where the stimulating electrode was placed). Recordings were performed using patch electrodes having an internal composition in mM of: KCl, 145; NaCl, 10; $CaCl_2$, 2; EGTA, 10 (yielding a free-Ca^{2+} concentration of 100 nM); MgATP, 2; dextrose, 10; and HEPES, 10; the solution was 300-320 mOsm, pH adjusted to 7.3-7.4. The input resistances of these electrodes ranged from 3-8 MΩ. A HEKA EPC-9/2 amplifier (Mahone Bay, NS, Canada) was used for all these experiments. Series resistance compensation was performed in all recordings. Acceptable recordings were those having an initial access resistance less than 20 MΩ that could be compensated by 40-70% (50-100 μs lag). The series resistance was monitored throughout the recordings; if it rose irreversibly above 20 MΩ the recording was terminated. Under these conditions and based on size of the currents monitored, filtering errors due to uncompensated resistance/capacitance were also negligible. Current clamp under these recording conditions produced negligible filtering errors. The bandwidth was set at 0-2 kHz. Recordings were performed in both voltage- and current-clamp mode.

Excitatory post synaptic potentials (EPSPs) recorded in current-clamp recording mode were evoked by stimulating the white matter with a concentric bipolar electrode (constant voltage, range of current 50-200 μA) using single pulses of 0.5 ms duration, delivered at 0.1 Hz. Stimulation was triggered by a computer. Threshold for a synaptic potential was determined and this was increased by 50% to minimise failures. In about 50% of the recordings no connection could be found, so these recordings were terminated. An average of 10 evoked EPSPs was used for each comparison. All data reported here are from cells that had a synaptic connection and were therefore considered neuronal (despite the fact that some in the lesioned untreated group had small or non-existent action potentials). All synaptic potentials were considered monosynaptic based on observed latencies of less than 1-2 ms. All recordings were done at resting membrane potential which was always greater than -60 mV.

Drugs and Chemical Reagents. 5-AP (DL-2-amino-5-phosphonovaleric acid) and DNQX were purchased from Precision Biochemicals Inc. (Vancouver, BC).

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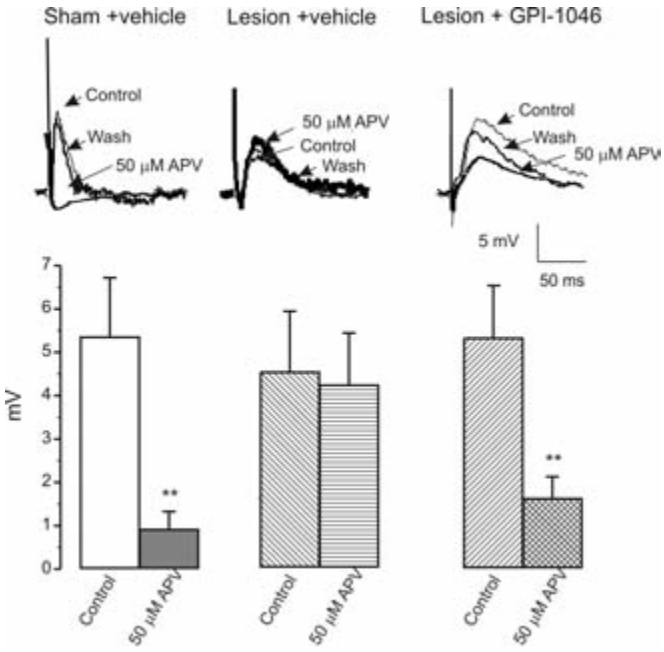

Figure 4: *GPI-1046 reverses the 6-OH-dopamine lesion-induced decrement in NMDA-mediated synaptic transmission. In sham-lesioned, vehicle-treated rats, 5-AP abolished the evoked corticostriatal synaptic potential (top left). In lesioned rats this potential becomes insensitive to 5-AP application (middle traces). However in GPI-1046-treated animals that had been lesioned, like in the sham-operated rat, this potential is sensitive to 5-AP (left traces), thus NIL treatment restores NMDA receptor-mediated synaptic transmission. The quantification of these results is shown below each set of traces.*

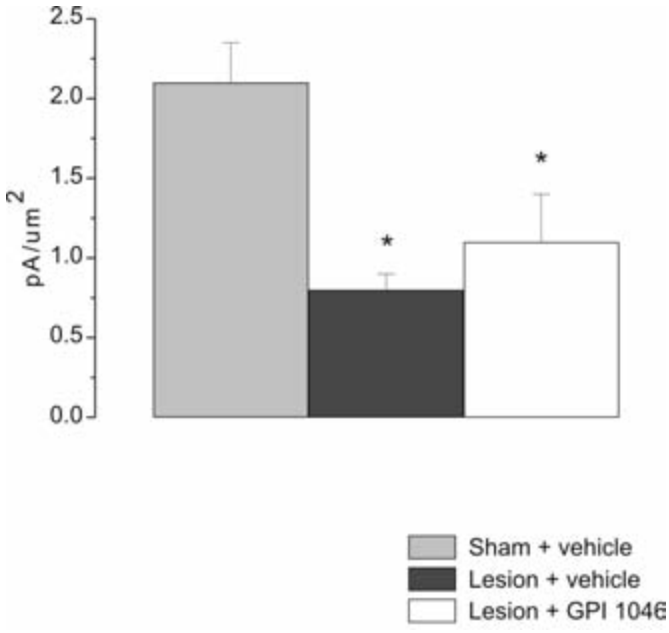

Figure 5: *6-OHDA lesioning reduces sodium channel current density but this is not reversed by GPI-1046.*

Analysis and statistics for electrophysiology. The amplitude of the synaptic potential was measured from the zero line to the peak of the synaptic potential recorded in current-clamp mode. All numerical data reported are represented as mean ± SEM. Comparison between means was calculated by t-test or ANOVA test, as appropriate. A "p" value of < 0.05 was regarded as statistically significant (two-tailed test).

Results

As NILS are known to associate with various intracellular messengers that may affect gene expression (heat shock proteins for example) we assayed mRNA levels using cDNA microarrays expressing ~15,000 clones (see Materials and Methods for details). Four groups of rats (Long Evans Hooded (LEH) strain, (n = 6) were prepared. 1) Sham-Vehicle 2) Sham + GPI-1046-treated 3) Lesion-Vehicle and 4) Lesioned + GPI-1046 treatment. A cross-over flip-dye design was used to compare gene expression profiles (as described in[20]). Our analysis revealed that about 50 different cDNAs were differentially expressed under the various conditions used here (see Appendix I for the complete list). Most hits (45 of 53) were expressed in the lesion-treated group. Many of the differentially expressed cDNAs were "expressed sequence tags" (ESTs) of unknown or unsure identity. We chose to validate sequences that were associated

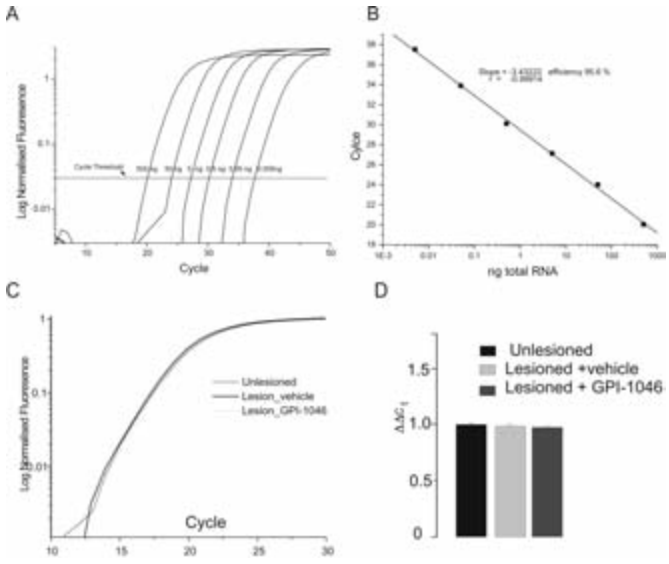

Figure 6: *QPCR analysis shows that NR-1 mRNA abundance is unaffected by lesioning or NIL treatment. Panel A shows the results of a standard curve experiment where a QPCR is run against the varying quantities of reverse-transcribed total cellular RNA. The cycle threshold is set 20X above background. In B, the results of this experiment show that an excellent relationship between total cellular RNA and cycle threshold was obtained. The slope of the relationship indicated that primer efficiency was about 96%. In C we show three amplification curves (for clarity) that indicate that no differences in mRNA abundance could be detected. The results of all experiments are summarised in Panel D. It appears clear from this that the increase in immunoreactivity (see Figure 7) was not associated with an increase in transcript abundance.*

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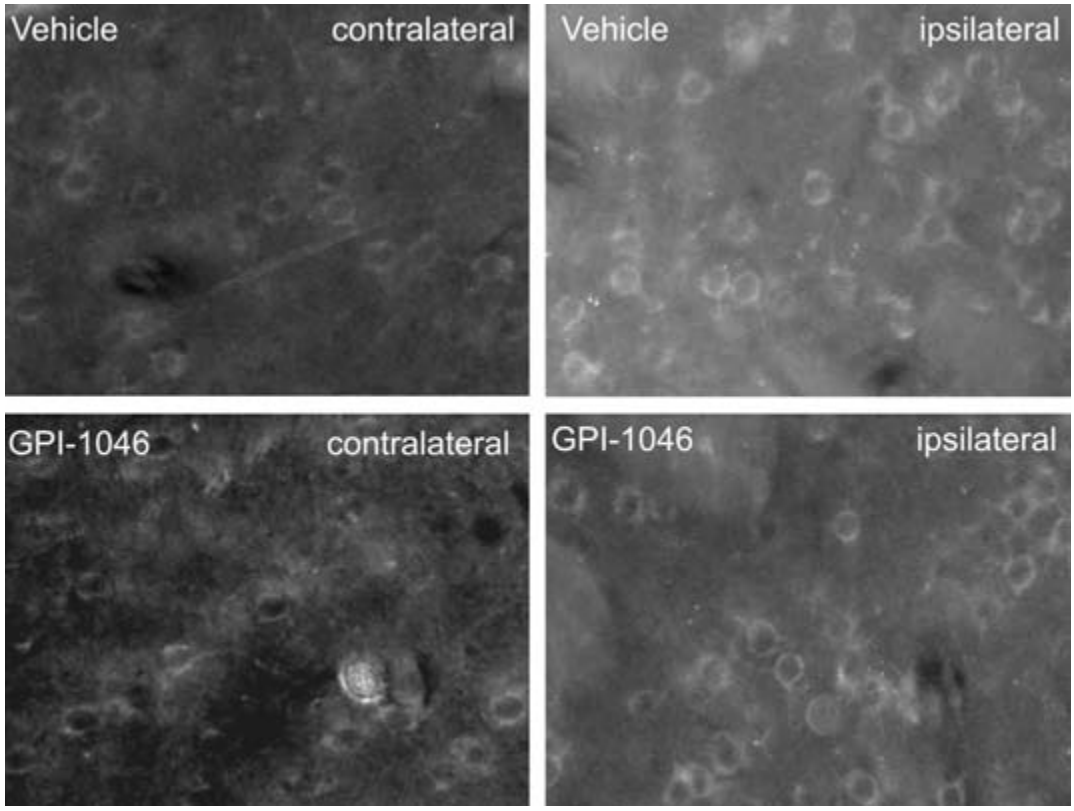

Figure 7: *Lesioning produces an apparent up-regulation of NMDA receptor immunoreactivity in striatum that has been lesioned (compare panel top left and right). GPI-1046 has no effect on this staining pattern (bottom panels left and right).*

with a known gene and that were deemed to be possible factors in regulating neuronal function. Our initial list was limited to ten hits (bolded in the Appendix). The validation was done by QPCR analysis of striatal mRNA from each treatment group. From this assay only one gene, presenilin-1 (PS-1), was subsequently found to be up-regulated in treated lesioned tissue (Figure 1). Specifically, we found that PS-1 mRNA was expressed at low levels in sham-lesioned and 6-OHDA-treated rats. However, in rats that had been lesioned and treated with GPI-1046, we found that PS-1 mRNA abundance was greatly increased. Figures 1A & B demonstrate the efficiency of the QPCR primers used to assay for PS-1 expression. In Figures 1C & D we show that PS-1 was up-regulated from low levels in the lesion vehicle group (LV) to levels that were about nine cycles earlier in the lesioned treated (LT) group (Figure 1C, LV: C_{tn} 15.3 ± 1.3 versus LT: 6.4 ± 1.6 $p < 0.001$). The higher C_{tn} in the lesioned vehicle group indicates less mRNA was expressed in the lesioned vehicle than in the lesioned treated tissue. Similar high-cycle thresholds were found for sham-treated and sham vehicle groups, indicating that PS-1 expression was low in these groups as well (see Figure 1D). These data suggest therefore that lesioning and GPI-1046 treatment was very effective in up-regulating PS-1 mRNA abundance.

In a separate group of rats, we performed immunocytochemistry in order to determine if PS-1 protein was up-regulated in striatal cells as the microarray and QPCR results suggested. The same four groups of animals were prepared (n = 4 per group). In Figure 2 we show confocal images taken with a 40X objective (N.A. = 1.0) in the striatum ipsilateral to the lesion. These data show PS-1 expression is low in sham vehicle treated and is decreased slightly in the sham treated group. In the lesioned vehicle group, PS-1 was expressed at very low (nearly undetectable) levels. However in lesioned GPI-1046-treated rats there was a robust expression of PS-1 in both cell bodies in the processes of the striatal cells. The immunoreactivity was localised to both the cytoplasm and the cell membrane. Quantitative image analysis showed that in lesion/GPI-1046 rats there was a ten-fold increase in receptor expression in comparison to the sham/vehicle groups (see methods for description of our quantification methodology). These results indicate therefore that lesioning/GPI-1046 treatment up-regulates PS-1 protein expression in striatum.

In order to determine if increased PS-1 expression was associated with re-innervation we measured tyrosine hydroxylase activity in the striatum. To do this we first quantified the size of the lesion by counting TH-positive cells in the SNc and then quantified the intensity of TH immunoreactivity in the corresponding striatum. This procedure allowed us to determine the relationship, if any, between extent of TH immunoreactive cell bodies in the SNC and the density of TH fibres in the

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striatum. Lesioning produced a 50-80% reduction in cell body density in both sham- and GPI-1046-treated rats. In non-treated rats a concomitant reduction of TH immunoreactivity in the ipsilateral striatum was correlated with lesion size ($r = 0.75$, $p < 0.01$). We predicted that GPI-1046 treatment would eliminate this relationship and cell body number would no longer correlate with TH immunoreactivity. However we found that in this strain of rat, GPI-1046 treatment had no effect on the TH immunoreactivity (Figure 3). This was surprising, as we and others have shown in Sprague Dawley rats that TH activity returns to near-baseline expression levels with GPI-1046 treatment [2,3]; (also see discussion). Thus GPI-1046 treatment and PS-1 expression was not associated with any changes in dopaminergic innervation.

In order to understand what functional consequences the up-regulation of PS-1 may have, we performed electrophysiological experiments to determine the effect of the lesioning and the impact of PS-1 expression on this state. We found previously that NMDA-mediated LTP was lost and restored by GPI-1046 treatment, post-lesion[3]. Others have found that the loss of PS-1 expression in conditional knock-out mice reduces NMDA receptor expression in hippocampus of mice[23]. Therefore we investigated the possibility that NMDA receptor function may be lost in lesioned mice and restored in mice first lesioned, then treated subsequently with GPI-1046.

We used the following protocol where we limited our recordings to cells whose cell body diameters were in the range of 8-12 μm. The voltage-dependent properties of the cells were examined by obtaining a V/I record in voltage-clamp mode in ACSF. Then in current clamp mode, we established whether a synaptic connection could be observed by stimulating the overlying white matter using a concentric bipolar electrode (usual stimulation intensity was 1.5 X threshold, providing that this intensity did not elicit an action potential, either antidromic or otherwise). If a synaptic connection was present, the Mg-free ACSF was switched to one containing the GABA receptor antagonist, bicuculline (20 μM). This pharmacologically isolated the input as being purely glutamatergic (in five separate recordings the addition of both DNQX and 5-AP completely abolished the remaining potential). In order to determine how much NMDA channel function was responsible for these synaptic potentials, we examined the effectiveness of 50 μM 5-AP (an NMDA receptor antagonist) to block this synaptic potential. In sham-lesioned rats both ipsilateral and contralateral to the injection site, striatal synaptic potentials were indistinguishable and were on average 5.3 ± 1.3 mV and 5.3 ± 1.2 mV, respectively $n = 7,8$ $p > 0.05$). These synaptic potentials were about 80% blocked by the application of 5-AP (sham = 84 ± 7% reduction, Figure 4). However, in striatum ipsilateral to the lesion, 5-AP had no effect on this potential, producing only a 9% ± 8% reduction in amplitude. In three recordings from lesioned and untreated rats, the subsequent application of DNQX completely abolished the 5-AP-insensitive synaptic potential. However in rats treated with GPI-1046, the sensitivity to 5–AP was largely restored (producing a 83 ± 25% reduction $n = 11$, $p < 0.03$). Thus, GPI-1046 treatment restored NMDA channel activity in these synaptic responses.

In order to determine if this effect was specific to NMDA receptors, we measured sodium channel density in voltage-clamp mode, as there is evidence that dopaminergic signalling may regulate sodium channel expression. This was done by measuring the peak sodium current, then calculating the current density based on the cell surface estimated from total cell capacitance. We also measured action potential height in current-clamp recordings. We found that the inward currents of neurons in lesioned striatum were much smaller than they were in sham-lesioned striatum (measured by current density: sham: 2.0 ± 0.2, lesion: 0.8 ± 0.1 pA/μm^2, $p < 0.001$, Figure 5). The difference in current density was correlated with lower spike amplitude (sham 81.4 ± 3.7 vs lesioned 58.0 ± 5.6 mV, $n = 19, 27$ respectively, $p < 0.01$). However, there was no change in threshold for action potential generation (sham 56 ± 3 mV vs lesioned 54 ± 4 mV, $n = 19, 27$ respectively, $p = 0.84$). Treatment with GPI-1046 had no effect on this lower current density or action potential height (0.9 ± 0.2, pA/μm^2 & 56.9 ± 6.7 mV, n= 19, 27, $p = 0.86$). Qualitatively, we also found that cells in lesioned striatum were much less excitable; in a few recordings (5 of 32), despite cells having normal resting membrane potentials and a synaptic connection, no action potentials were produced. Overall, and as hypothesised, sodium current density was reduced after lesioning, but GPI-1046 treatment had no effect on this decrement in activity.

These data indicated that NMDA receptor function was restored independent of dopamine re-innervation and was correlated to the increase in PS-1, similar to observations made by Saura et al (2004)[23]. This study also showed that the alteration in synaptic localisation was independent of NR-1 mRNA/protein expression, which is the subunit essential for NMDA receptor function. Thus we wished to see if NMDA receptor expression was altered here. The results in Figure 6 using QPCR analysis show that NR-1 subunit mRNA abundance was not associated with lesioning or GPI-1046 treatment (and PS-1 expression). Thus the losses and/or restorations of NMDA receptor function were not associated with mRNA levels. We also checked to see if NR-1 protein expression might be altered after lesioning and/or GPI-1046 treatment. Using anti-NR-1 antibody, we probed for NR-1 subunit expression in the same brains as those used for the electrophysiological and TH immunohistochemical experiments described above. As shown in Figure 7, NR1 protein expression was apparently up-regulated in lesioned rats. However, this increased expression was unaltered by GPI-1046. So, in spite of an up-regulation in protein expression after lesioning, the striatal cells still lost NMDA channel function for the synaptic connections between the cortex and striatum.

Discussion

Our data show that: 1) GPI-1046 treatment increases the expression of PS-1 mRNA and protein; 2) GPI-1046 did not restore dopaminergic innervation of the striatum in the Long Evans hooded strain of rats unlike that previously observed for Sprague Dawley rats[2,3]; 3) synaptic NMDA receptor function was lost due to lesioning but was restored by GPI-1046 treatment; 4) 6-OHDA-induced denervation of the striatum induces a down-regulation in sodium current and this loss was unaffected by GPI-1046 treatment; and 5) after lesioning, NMDA receptor NR-1 subunit mRNA expression is static while protein expression increases, but these expression profiles are independent of innervation and GP1-1046 treatment. Our

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interpretation of these observations is that GPI-1046 restores NMDA channel function through the up-regulation of PS-1 expression which has been previously shown to be critical for NMDA function in dentate granule cell synapses[23]. These data imply that NMDA receptor-mediated synaptic function can be restored in the striatum independent of the dopaminergic mechanisms that normally contribute to its maintenance.

It was surprising that GPI-1046 failed to restore dopaminergic activity or counteract the rotational behaviour induced by amphetamine and/or apomorphine injection in the Long Evans hooded rat. We and others have found GPI-1046 reversed these outcomes in Sprague Dawley rats. However, these observations are similar to those that have previously been observed elsewhere - for example C57 strains of mice do not respond to NIL treatment but the BALB/cByJ and CD1 strains do (see review)[19]. Thus as we have previously pointed out, GPI-1046 efficacy seems to be highly species/strain dependent. Nevertheless, GPI-1046 did re-establish a near-normal level of corticostriatal excitatory synaptic transmission. This recovery was not sufficient to reduce the stereotypical rotational behaviour, indicating that the restoration of dopaminergic function is critical to eliminate this motor defect.

We also found that the loss of dopamine innervation was associated with reduced sodium channel function. This finding is analogous to observations made in the locus coeruleus where chronic opiate and dopamine stimulation up-regulates sodium channel function through cAMP-dependent pathways[24]. Presumably, the loss of dopaminergic input here down-regulates cAMP-stimulated pathways, reducing sodium channel density/function.

Due to the methodology used here we were unable to assess directly whether LTP could be induced although reasonably it would be expected that loss of NMDA receptors should be sufficient to prevent the induction of that form of synaptic plasticity. However, LTP is also completely blocked by D1 but not D2 antagonists, and mice lacking the D1 receptors do not support LTP[3,25]. Thus it is unlikely that LTP was re-established in these rats despite the recovery of NMDA receptor function. Parenthetically we should add that the absence of LTP likely does not account for the lack of motor recovery, as D2 receptors seem to be more important for the regulation of turning behaviours[26].

After lesioning of the SNc one study found that NMDA receptor expression increased[27], while two others have shown that NMDA receptor mRNA and protein expression does not change[27-29]. This discrepancy may be accounted for by the differing methodologies used. Fan et al (1999)[29] showed that a loss of NMDA receptor expression could be detected from the membrane-bound fraction, but when this fraction and the cytosolic one were pooled, there was no difference in total protein content. Here we found that mRNA expression is constant but protein levels may increase (as evidenced by the increased immunoreactivity). It is not clear to us what accounts for our observations but our data and those of others suggest that NMDA receptors, although plentiful, are not found in the membrane (or synapses). Thus the loss of NMDA-receptor mediated synaptic transmission does not appear to be correlated with protein or transcript abundance.

This suggests of course that the loss of NMDA-mediated transmission is due to the inability of the cells to traffic NMDA receptors into synaptic sites. Indeed cAMP-mediated plasticity of NMDA expression had been demonstrated previously[30,31]. These studies showed that up-regulation of PKA activity phosphorylates NDMA receptors and stimulates their localisation in synaptic sites.

Also PS-1 is implicated in the formation and maintenance of synaptic sites[32]. Importantly, our findings appear to corroborate a report where PS-1 has been shown to be essential in NMDA receptor localisation at synaptic sites[23]. This report showed that in conditional knock-down mice, the loss of PS-1 produced a number of changes in the hippocampus that may explain our findings. The loss of PS-1 expression in these mice resulted in NMDA receptor function being lost with a concomitant loss of LTP, yet, NMDA receptor expression was unchanged. The loss of function was ascribed to the absence of NMDA receptors in the synaptoneurosomes. Furthermore, this loss was specific to NMDA, as AMPA-mediated synaptic transmission was unaffected. The same study demonstrated that PS-1 was co-immunoprecipitated with the NR-1 subtype in the synaptosomal preparations. A more recent report has suggested that pre-synaptic mechanisms may be at play as well[33]. That study found that the loss of presynaptic expression of presenilin-1 reduced glutamate release, and that in turn this reduction decreased the probability of NMDA channel activity. The additive effect of these alterations was the prevention of LTP. While we have no mechanistic data that would resolve these two views, our observations appear to support the notion that PS-1 controls NMDA receptors, and synaptic function is restored by drug-induced expression of PS-1.

Although it seems plausible that PS-1 may enhance the CREB/CBP-mediated transcription that is lost due to 6-OHDA lesioning, we have been unable to identify any potential link between NILs activity and PS-1 expression. The former are thought to bind to various transcription factors[34,35] and heat-shock proteins (FKBP-52 is also known as HSP-56[19]). One study has indicated that the primary target of GPI -1046 for eliciting neurotrophic effects may be FKBP-38[34], but no link between this or any other protein and PS-1 gene regulation has yet been demonstrated. Furthermore, a mapping and analysis of putative PS-1 human, chicken, rat, and mouse promoter sequences (using software Conreal:)[36,37] shows no consensus of transcriptional binding sites that can be linked to any transcription factors known to interact with NILs. Thus, the mechanism by which GPI-1046 up-regulates PS-1 expression remains unknown at this time.

Although mutant forms of PS-1 have been convincingly implicated in various forms of neurodegeneration[38], the role of the native form is not completely appreciated[32]. However, it has been suggested that some common pathways involving presenilins may be involved in both Parkinson's disease and dementia[39]. Indeed, one report has shown that a mutant isoform (G217D) has been associated with tangles that are found in the cerebral cortex and caudate[40], the latter being associated with Parkinsonian motor deficits. In rat, PS-1 is expressed in moderate amounts in the striatum along with APP[41] but its role in this area is largely unexplored. Nevertheless, PS-1 is essential for normal development as homozygous PS-1 knockout mice do not survive beyond E18[42]. In contrast to the toxicity observed when mutated forms are expressed, over-expression of wild type

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PS-1 has been shown to be relatively benign, even conferring positive outcomes. For example, it has been shown to prevent apoptosis[43], promote cell survival during development[44], and reduce excitability by enhancement of the delayed rectifier current when it is transiently-transfected into neurons[45].

We should also note that the mRNA levels of PS-1 are only different in the lesion treated group, but the levels of PS-1 protein expression appear to fall in the lesion group. Thus mRNA expression is not correlated with PS-1 protein expression in the lesion group; we are unsure as to why this is case. One explanation may be related to the fact that the stable expression of PS-1 is also dependent on the expression of other members of the γ-secretase protein family (nicastrin, Aph-1 and PS-2, see review:)[46]. This was first recognised in studies that attempted to up-regulate PS-1 expression by increasing mRNA abundance but no effect on protein expression was observed. This suggests of course that some other γ-secretase proteins may decrease in expression due to the lesioning, leading to the loss of PS-1 expression. This implies that treatment by GPI-1046 may induce the up-regulation of another member of this family. However, no study exists that has addressed the idea that Parkinson's or lesioning affects the expression of any protein in the γ-secretase family, and so this idea warrants further study.

Conclusion

In sum, this study has shown that lesions of the substantia nigra lead to a selective loss of NMDA receptors on striatal neurons that receive monosynaptic connections from the cortex. This loss is reversed by GPI-1046 in the absence of re-innervation by dopaminergic fibres. The loss of NMDA receptor-mediated synaptic transmission likely contributes to the disappearance of corticostriatal LTP, but its reversal is likely not sufficient for the restoration of normal motor behaviour. These changes in NMDA receptor function are correlated with up-regulation of PS-1 mRNA and protein expression. Although a modest increase PS-1 expression was seen due to GPI-1046 treatment alone, the combination of NIL treatment and denervation induced the most robust up-regulation. Finally we should note that although the results imply a central role for PS-1, we have not been able to follow-up on the role of the other differentially expressed genes (unknown) in our microarray data. Thus we cannot rule out the possibility that some other transcript may play a critical role in the pharmacological outcome documented. Follow-on experiments that further investigate and identify the mechanism(s) of this highly novel drug effect are the next step in these investigations.

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Appendix

Clone	Gene/EST Identity (if known)	Genebank Seqeunce
H3129G02	**Serine palmitoyltransferase, long chain base subunit 1**	**BG074002**
H3029C08	**Pax transcription activation domain interacting protein**	**BG065280**
H3074G01	**Hsp70-1> (Hsp40) homolog, subfamily A, member 3**	**BG069284**
H3027B08	EST similar to JC5921 non-selective cation channel	BG065083
H3100B02	**Synaptotagmin 11 (Syt11), mRNA**	**BG071533**
H3116C08	EST	BG072898
H3037D04	EST	BG065963
H3085D04	EST	BG070256
H3140C07	EST	BG074861
H3150D02	**presenilin 1**	**BG075684**
H3095H09	ESTs	BG071204
H3108G09	Ctbp1 -> C-terminal binding protein 1	BG072281
H3154D09	Tyrosinase (Tyr), mRNA"	BG076005
H3091A09	No items found.	none
H3051C10	EST	BG067163
H3030C07	myodystrophy -> myd -> Large -> like-glycosyltransferase	BG065368
H3085F03	Pabp-interacting protein 2 (Paip2-pending)	BG070278
H3012B06	Protein phosphatase 1, catalytic subunit	CK334256
H3005H06	Protocadherin alpha 11 (Pcdha11), mRNA	BG063331
H3035H08	ESTs	BG065847
H3025H03	Mitochondrial translational initiation 2 (Mtif2), mRNA	BG064965
H3133C02	RIKEN cDNA 5730427N09 gene	BG074286
H3091C07	Spermatogenesis associated glutamate 4b (Speer4b)	BG070798
H3043E06	Pyruvate dehydrogenase E1alpha subunit	BG066484
H3070C07	ATPase, H+ transporting, V1 subunit F (Atp6v1f)	BG068877
H3016F06	"Myosin IC (Myo1c), mRNA"	CK334323
H3135G07	Ppox -> Ppo -> protoporphyrinogen oxidase	BG074490
H3037H08	Mov-10 -> Mov10 -> Moloney leukemia virus 10	BG066012
H3042E01	D1Ertd228e -> DNA segment	BG066391
H3133A08	EST	BG074270
H3010H09	Tripartite motif protein 7 (Trim7), mRNA	BG063811
H3087A04	Hydroxysteroid dehydrogenase-2, (Hsd3b2)	BG083324
H3151E02	EST, Weakly similar to I49130 reverse transcriptase	BG075769
H3121A02	**Dispatched homolog 1 (Drosophila) (Disp1-pending)**	**BG073297**
H3138C01	pp90rsk> ribosomal protein S6 kinase, polypeptide 2	BG074699
H3143D08	Stx5a -> 0610031F24Rik -> Stx5 -> syntaxin 5a	BG075109
H3101G06	Cell division cycle associated 1 (Cdca1), mRNA	CK334739
H3041E08	1500011H22Rik -> RIKEN cDNA 1500011H22 gene	BG066318
H3041F10	**Neuraminidase 3 (Neu3), mRNA**	**BG066328**
H3062C07	Polyhomeotic-like 3 (Drosophila) (Phc3), mRNA	BG068148
H3130B03	ESTs	BG074037
H3122B07	**Heat-responsive protein 12**	**BG073398**
H3131H12	Rpl37a -> ribosomal protein L37a	BG074193
H3158H09	EST, Highly similar to phosphoinositide 3-kinase	BG076369
H3028A09	Bach2 -> BTB and CNC homology 2	BG065166
H3117G08	**Caspase 8 associated protein 2**	**BG073027**
H3077H01	Ring finger protein 38 (Rnf38), mRNA	BG069576
H3089F04	RIKEN cDNA 0610038K03 gene (0610038K03Rik)	BG070648
H3109E04	Carbonic anhydrase 9 (Car9)	BG072347
H3104E07	Calcitonin activity modifying protein 2	BG071909
H3154E07	**Growth arrest specific 6**	**BG076011**

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