Neuroprotection in Parkinson's Disease: a Multi-directional Genetic Strategy for Maximum Protection of Dopaminergic Neurons against Parkinsonian Toxicity

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Abstract

The complex biology of Parkinson's disease and the obscure mechanism of dopaminergic cell death in the course of the disease indicate that multiple intracellular pathways and numerous crucial elements contribute to the demise of these neurons. Therefore, multi-factorial approaches would more likely confer long-lasting survival and potentiate the biological function of dopamine neurons. We are proposing a multi-directional strategy to protect dopamine neurons against parkinsonian toxicity that involve transcription, anti-oxidant and neurotrophic factors. Specifically, Nurr1 an important DA transcription/ anti-inflammatory factor, glutathione peroxidase-1 an anti-oxidant enzyme (GPX-1) and glial cell line-derived neurotrophic factor (GDNF) a potent neurotrophic factor have all shown their capacity for dopaminergic neuroprotection. A model we are proposing is based on dopamine neuron-astrocyte-microglia co-culture that will supply all three factors in a tripartite fashion accelerating gene-to-gene and cell-to-cell cross-talks for synergy. While microglia will overexpress Nurr1, astrocytes will act as minipumps to secrete GDNF into the medium to act on GPX-1-overexpressing dopamine neurons growing within their proximity. The neurons will ultimately be exposed to the parkinsonian neurotoxin 6-OHDA and tested for their improved survival rate in vitro and in vivo, their integration capacity to neural network and their physiological function in the midbrain circuitry.

Keywords
Neuroprotection, GDNF, Nurr1, GPX-1, Parkinson
Introduction

Neurodegeneration and PD

Neuronal cell death is the main hallmark of neurodegenerative disorders. PD is characterized by degeneration of midbrain dopaminergic (DA) neurons (1) and disintegration of their nigro-striatal (SN-ST) fibers (2, 3). There is no known factor that initiates DA neuron death in PD, but environmental toxins and mutations in a number of genes are suspected causes (4).

Oxidative and nitrosative stress play an important role in PD development and progression (5). In general, multiple sources can cause increased reactive oxygen species (ROS) responsible for oxidative stress in the CNS (reviewed in ref. 6). Among these sources are: a) mitochondrial dysfunction including complex I inhibition leading to ATP depletion (7) that further increases ROS levels outside mitochondria, b) non-neuronal factors specially the hallmarks of neuro-inflammation secreted by activated microglia and astroglia (8) that selectively destroy DA neurons (9), c) free dopamine that enhances generation of H2O2 and DA quinones capable of changing protein structure (10-12) and that disturbs cellular redox status and damages DA and non-DA neurons (12-14), d) GSH depletion that causes unlimited generation of ROS and damages mitochondrial complexes (15), e) protein aggregation such as synuclein aggregation and ubiquitination.

Nitrosative stress is mainly due to S-nitrosylation (SNO) of specific cystein thiol groups during which nitric oxide (NO) reacts with thiol to form an SNO-protein and thus regulates protein activity (16, 17). SNO occurs in the structure of important proteins such as parkin and reports indicate widespread S-nitrosylation of parkin in PD brains (18, 19). By modifying its structure, SNO compromises the neuroprotective activity of parkin and causes ubiquitin-proteasome system (UPS) to dysfunction (19, 20). These changes could have profound implications in PD since DA neurons are vulnerable to UPS dysfunction. Overall, nitrosative and oxidative stress that cause parkin dysfunction may link ROS generation to UPS dysfunction, protein aggregation and DA neuron death in sporadic PD.

General view of neuroprotection

Protection of neurons from dying is an effective strategy to challenge neurodegeneration (21). Potent elements including neurotrophic factors, anti-apoptotic molecules and anti-oxidant enzymes have been individually tested for their neuroprotective capabilities. However, a comprehensive mode of neuroprotection seems unlikely before their joint applications can potentiate their synergistic effects.

GDNF: glial cell line-derived neurotrophic factor (GDNF) promotes survival and axonal growth of mesencephalic DA neurons (22). The molecule has been studied to the extent that its current applications span from cultured cells to PD animal models (23, 24), and, taking its clinical trials into account, from bench to bed (25-30). GDNF ameliorates motor deficits and reduces brain damage in animal models of PD (31-33). It also protects DA neurons already committed to death (32), and further induces renervation of their residual fibers and so assists reconstitution of the demolished SN-ST pathway (33). In human brain, Love et al. have produced direct evidence for GDNF-mediated DA neuron sprouting (34). Furthermore, direct intra-striatal administration of GDNF improves daily living and dopamine metabolism of PD patients (25, 28).

GPX-1: GPX-1 plays an important role in protecting neurons against oxidative damage by reducing levels of hydrogen peroxide and so production of highly reactive hydroxyl groups (35). The neuroprotective role of GPX-1 against ROS-mediated cytotoxicity has been demonstrated by both in vitro and in vivo studies on GPX-1 transgenic animals (36, 37). GPX-1 functions by preventing disturbances in redox status and reduction of lipid, protein and NADH/NADPH oxidation (38, 39). Expression levels of GPX-1 are reportedly increased in PD brain (40) leading to attenuation of parkinsonian toxicity in DA neurons both in vitro and in vivo (41-43).

Nurr1: beside its developmental function in DA neuron genesis and maintenance (44), Nurr1 has been implicated in neuroprotection as evident from its direct interaction with vasoactive intestinal peptide a protecting neuropeptide (45). Other reports indicate convergence between DA fate-determinant Nurr1 and neurotrophic factor GDNF and its receptor Ret at some point in their signaling pathways (46, 47). Both Nurr1 and GDNF have shown to be life-span supporting elements of DA neurons (48, 49).

Nurr1 has recently been implicated in the attenuation of inflammatory response in microglia and astrocytes (50). This study showed up-regulation of Nurr1 expression in microglia and astrocytes activated by lipopolysaccharides (LPSs) as inducers of neuro-inflammation that leads to the loss of DA neurons in rodents (51). On the other hand, it showed loss of Nurr1 function potentiates and prolongs inflammatory response to LPS (50).

Shortcomings of protecting factors

The neurotrophic and anti-oxidant elements have been unable to confer sufficient long-lasting protection to DA neurons in PD brains and, in some cases, have produced side effects. For instance, continuous supply of GDNF or high-level constitutive overexpression of its cDNA might lead to reduced levels of tyrosine hydroxylase (TH) protein in the preserved striatal dopamine terminals and aberrant sprouting of TH+ neurons in the striatum (ST) and SN regions.
Clinical trials have further shown that GDNF alone may not be able to sustain improvement in motor behaviors (26).

GFP-1 and Nurr1 can only partially support the anti-oxidant and anti-inflammatory defense systems. This is due to the multiplicity and diversity of signaling pathways contributing to increased ROS production in PD brain (54), which bars single-agent approaches from fully inhibiting excessive ROS production.

A more puzzling contributor to the insufficient protection is the unknown initiators of DA neuron death that triggers PD onset. Although environmental toxins have been traditionally blamed as prime suspects, endogenous toxicity led by genetic mutations discovered in recent years (reviewed in 55) has to be taken to account too. Findings in both directions have led to a hypothesis which suggests possible interactions between environmental factors such as infection and common but less penetrant susceptibility genes as the prime initiators of DA neuron death leading to the onset of sporadic PD (4, 56).

Hypothesis/Idea

Based on the knowledge currently available on the pathophysiology of PD, we are proposing a combined strategy that we believe is more likely than single-agent approaches to succeed in preventing or halting neurodegeneration and compensating for the lost motor functions if applied in vivo. Three cell types and three effectors constitute the basis of our strategy (Figure 1). Multiple interactions occur among these parties in nature that potentiate synergistic effects for survival (Table 1). Our strategy will benefit from these natural interactions by directing them toward safeguarding DA neurons from any life-threatening insults.

The strategy in vitro: astrocytes and microglia will be transduced with recombinant lentivirus stocks carrying coding sequences, respectively, for GDNF and Nurr1 (Figure 2-In vitro). For spatiotemporal regulation of gene expression, the coding sequences can be positioned under the control of tetracycline-inducible systems that work well in both DA neurons under the TH promoter (57) and astrocytes under the GFAP promoter (58).

A co-culture system will then be established between a mixture of transduced microglia and astrocytes, on one hand, and TH+ neurons of various sources transduced to express GPX-1, on the other. These neuronal sources will include TH+ cell lines and primary ventral mesencephalic culture from day 12-13 mouse or rat embryos. The effect of inducing factors expressed by all parties on the survival of DA neurons against 6-OHDA toxicity will be examined by standard cell viability assays. The neuroprotective effects of the mixed culture co-secreting GDNF and Nurr1 will be compared with those cultures secreting only one of them: transduced microglia + plain astrocytes and vice versa.

The strategy in vivo: based on the observations made in culture dish, transduced microglia and astrocytes will be selected for similar evaluation in animals. PD animal models can easily be made that contain 6-OHDA-mediated lesions and used for the experiment. Selected cell groups will be stereotaxically injected into the animal's ST using coordinates from bregma. The injected cells will be examined for their survival, secretion of inducing factors and protection of DA neurons in the region (Figure 3-In vivo). In this setting, lentiviruses harboring GPX-1 will be injected to DA neurons in the SN. All lentivirus constructs will be equipped with a fluorescent marker such as GFP, Yred or YGFP to help the cells to be localized and traced upon injection. Cell type-specific markers GFAP, OX-42 and TH will also be applied in immunohistochemical analyses to identify astrocytes, microglia and DA neurons, respectively.

Evaluation of the Hypothesis/Idea

Evaluation In Vitro

1. Gene expression: it will be tested at the mRNA level by RT-PCR and at the protein level by immunocytochemistry or Western blot analysis using protein-specific antibodies. In the case of GDNF, there is a reliable ELISA kit to detect secreted levels of the protein in the supernatant.

2. Virus production: this will be monitored by a sensitive ELISA test for HIV-1 capsid protein p24 that is routinely used for HIV-1 patients in clinic and works well for detection of mature recombinant lentiviruses. The expression levels of reporter fluorescent genes such as GFP co-expressed with candidate proteins measurable by flow cytometry or real-time PCR can be relied upon to determine virus titer.

3. Activity of genes: GDNF-mediated cell survival in the presence of parkinsonian neurotoxin 6-OHDA can be determined by MTT and other viability assays as we have reported (58). As for GPX-1, there exist special assay kits in the market that measure its biological activity. Nurr1 activity in DA differentiation can be measured by appearance of dopamine-synthesizing enzymes such as TH or aromatic amino acid decarboxylase (AADC).

4. The anti-inflammatory activity of Nurr1: microglia and astrocytes will be activated by addition of bacterial LPS (Figure 2-In vitro). LPS induce levels of transforming growth factor alpha (TNFa), interleukin-1 beta (IL-1β) and inducible nitric oxide synthase (iNOS) (50). However, the strong presence of Nurr1 in the cells should prevent this activation process. Therefore, Nurr1 activity in induced microglia or astrocytes can be determined by measuring expression levels of the above elements as hallmarks of inflammation.
5. Synergistic effects: conceivably, an experimental option that has all components in place must confer higher protection against parkinsonian toxicity than controls where one or two of the protecting elements are missing. Synergistic effects between GDNF-GPX-1, GDNF-Nurr1, Nurr1-GPX-1 and GDNF-Nurr1-GPX-1 will be tested by transducing the cells with each setting both in culture dishes and in 6-OHDA-mediated lesioned animal’s midbrain (see below). To be specific, serial titers of virus stocks will be applied for target cell infection. Where necessary, dosage control will be achieved using inducible systems of expression as we have reported (57). Moreover, a tri-partite contribution will be tested by relying on appropriate controls where the parties will be deleted one at a time. This will allow the causal relationships and in particular synergistic effects to be dissected and stepwise examined. For instance, the impact of GDNF over-expression on reduction of free radical levels leading to increase survival rate for DA neurons will be compared to the co-presence of GDNF-Nurr1, or GDNF-GPX-1, etc.

**Evaluation In Vivo**

The in vivo test requires the presence in the scene of microglia-astrocytes culture system outlined above. Therefore, the mixed astro-microglial culture virally transduced to secrete Nurr1/GDNF will be injected to the ST in a PD animal model while the animals will receive GPX-1 lentivirus in their SN. PD models can readily be created by administering 6-OHDA or MPTP as parkinsonian neurotoxins to the ST to introduce one-sided lesions into the brain. Animals will be divided into two groups: 1) one group will be sacrificed in appropriate time points to prepare sections from their SN and ST regions. Evaluation of gene expression and the biological activities of the protecting factors will be carried out by analyzing these dissected brain sections via immunochemical analyses and the procedures outlined for in vitro.

Individually, GDNF levels will be measured by ELISA while its activity on the survival of TH+ neurons and renervation of their SN-ST fibers will be traced by immunostaining as well as cell count. GPX-1 will be assayed for its activity by measuring 6-OHDA-mediated ROS elevations, expression levels of inflammation hallmarks (listed above) will be monitored before and after injection of Nurr1-overexpressing microglia. A comparison will be made by including control animals receiving LPS injection instead of 6-OHDA (Figure 3-In vivo) (50).

To monitor synergistic effects of the three elements, the above evaluations will be carried out on test animals receiving all three protecting elements, i.e. GDNF, GPX-1 and Nurr1. The data from this test will be compared with controls where only one or two of the three factors are supplied by the injected culture (see evaluation in vitro, section 5).

2) A second group of animals will be kept to use for behavioral analysis. In this case, a routine amphetamine-induced rotational test is available that will be carried out on both test and control animals to compare the final effect on motor behavior.

**Discussion and Conclusion**

Neuroprotection is considered an effective strategy to challenge neurodegenerative disorders including PD during which DA neurons die. Here we presented a hypothetic strategy for maximizing protection of DA neurons from degeneration. A wealth of reports points to oxidative/nitrosative stress and neuro-inflammatory damages as the major players in DA neuron degeneration in PD brains. Factors known as neural protectants hold promise to support neuroprotective therapy but from clinical perspective, they are unable to deliver their promise when applied individually. This problem will likely be resolved by amplifying synergistic effects that are common among signaling molecules as are the convergent points between their signaling pathways.

As shown in Figure (1) and outlined in Table (1), the tripartite cells and molecules are in high-level communication with one another. In such an interactive environment several points of synergy develop among the parties. Therefore our strategy will maximally benefit from these dynamic interactions due to the active presence of interacting cells/molecules.

An interactive approach is indeed much needed for nigral DA cell bodies because as they proceed to death in PD brain, their SN-ST fibers are severely dismantled, demanding highly protective measures to restore them. Studies indicate retrograde GDNF travel from the ST back to the SN and so it mediates higher protection when overexpressed in the ST compared to its overload in the SN (32). Therefore, we believe our proposed experiments would not only safeguard the neuronal cell bodies in the SN (by GPX-1) but also will guarantee restoring the SN-ST pathway (by GDNF-Nurr1 partnership) damaged in the course of PD progression.

Astrocytes are advantageous biological minipumps to deliver trophic and other supporting factors to neurons. On one hand, GDNF-overexpressing astrocytes protect DA neurons against parkinsonian toxicity as shown by us (58) and many others. They can also provide a context for synergistic effects by cross talking with neurons, as we have shown that GDNF and glutathione can synergistically protect DA neurons (58). Astrocytes, on the other hand, are crucial for specifying and maintaining the phenotypes of midbrain DA neurons (59). Furthermore, it is known that pro- or anti-inflammatory response by microglia is primarily sensed by astrocytes (60). So, microglia-astrocytes mixed population will likely facilitate cross-talks.
between the two cell types and potentiate strong anti-oxidant and anti-inflammatory signals in support of anti-parkinsonian defense for DA neurons. Overall, we presume that astrocytes will play a pivotal role by carrying messages from microglia overexpressing Nurr1, themselves by secreting GDNF as a key DA cell protectant, and synergizing these two elements with GPX-1 overexpressed by DA neurons.

We have hypothesized that co-upregulation of GDNF and GPX-1 will offer wider coverage for DA neurons (61). In fact, our very recent observations indicate that the simultaneous supply of GDNF by astrocytes and of GPX-1 by DA neurons protect these neurons against parkinsonian toxicity wider than by either agent alone (manuscript in preparation). This is in line with the finding by Chao and Lee that GDNF increases GPX-1 activity and, in return, an active GPX-1 enhances protection offered by GDNF (62).

The Nurr1-GDNF link has already been established, as outlined above (See Table 1). Co-presence of these two elements in the extra-neuronal environment will enhance neuroprotection by potentiating GDNF/Ret signaling pathways (63). On the other hand, DA neurons are sensitive to microglial injury for two reasons (Table 1): 1) low levels of anti-oxidant defenses: inclusion of GPX-1, GDNF supports this defense and so inhibits neuronal damage by microglia, 2) inflammatory response: Nurr1 will deter this injury by repressing pro-inflammatory gene expression (50).

In conclusion, we believe Nurr1, GDNF and GPX-1 as three of the most effective protecting factors in the brain will more specifically and effectively support target DA neurons than when they act alone. The ultimate outcome of genetic and cellular tripartite participation outlined in our strategy will be DA neurons protected in multiple directions: by supporting pro-survival functions of GDNF on nigral cell bodies and their SN-ST fibers, by deterring microglial injuries with Nurr1/GDNF alliance, and by empowering the anti-oxidant potential of GPX-1 to challenge excessive free radicals that float in neuronal cytosolic environments. Our suggested strategy will not only benefit from the additive and synergistic effects of these molecules but also will mimic the physiological scenario where cross-talks between microglia and astrocytes will accelerate neuronal protection.

Acknowledgement

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Overview Box

**First Question: What do we already know about the subject?**
Neuroprotection is considered one of few effective options for PD therapy. All three elements involved in this hypothesis have been tested positively for their neuroprotecting activities.

**Second Question: What does your proposed theory add to the current knowledge available, and what benefits does it have?**
The individual elements do not provide comprehensive protection against toxicity from oxidative stress and neuroinflammation. Our theory is based on the converging points in signaling pathways (likely cross-talks between astrocytes and microglia) used to accelerate the synergistic effects of these elements applied in combination.

**Third question: Among numerous available studies, what special further study is proposed for testing the idea?**
Acceleration of neuroprotection via cell-to-cell and gene-to-gene communications: we are proposing a co-culture system that will provide a cross-talk between astrocytes and neurons, and between astrocytes and microglia. It will also provide a cross-talk at molecular levels between the protecting elements mentioned above.
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<th>Type of Interaction</th>
<th>Arrow # on Fig.1</th>
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<td><strong>Cell to cell</strong></td>
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<td><strong>Astrocyte-DA neurons:</strong> astrocytes intimately support neuronal survival and maintenance by secreting growth factors and anti-oxidant enzymes</td>
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<td><strong>Microglia-DA neurons:</strong> activated microglia release inflammatory mediators such as NO, TNFα, IL-1β that can cause DA neuron death</td>
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<td><strong>Astrocyte-Microglia:</strong> Inflammatory factors secreted by microglia are sensed primarily by astrocytes that play an intermediate role between microglia and neurons</td>
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<td><strong>Gene to cell</strong></td>
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<td><strong>GDNF-DA neurons</strong></td>
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<td>GDNF induces anti-apoptotic Bcl2/Bclx by increasing P13K expression.</td>
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<td>GDNF neurotrophic response is mediated by CK2, a molecule that is involved in vital activities: signal transduction, protein synthesis, cell-cell adhesion, gene transcription that all are involved in neurotogenesis/cell survival.</td>
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<td>GDNF/GFRα1/Ret complexes interact with src kinase that plays roles in neuroprotection and neurotogenesis</td>
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<td>In the absence of Ret, GFRα1 associates with NCAM, Fin and Fak involved in cell adhesion, migration and intercellular communications.</td>
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<td>GDNF induces MAPK/ERK pathway that inhibits NMDA-induced cell death</td>
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<td><strong>GDNF-Microglia</strong></td>
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<td>By interacting with its receptors Ret/ GFRα1 on cell surface, GDNF improves microglial survival and phagocytic activity.</td>
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<td>It reduces NO synthesis and increases SOD production by microglia.</td>
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<td>Microglial activation induces GDNF expression leading to DA sprouting and DA neuron survival.</td>
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<td><strong>Nurr1-Microglia</strong></td>
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<td>Nurr1 represses pro-inflammatory genes in activated microglia and astrocytes</td>
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<td><strong>Nurr1-DA neurons</strong></td>
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<td>Nurr1 deters neuroinflammatory damage to DA neurons and prolongs their survival.</td>
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<td><strong>GPX-1-DA neurons</strong></td>
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<td>GPX-1 reduces free radical levels and so inhibits oxidative damage to DA neurons.</td>
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<td><strong>Gene to gene</strong></td>
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<td><strong>GDNF-Nurr1</strong></td>
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<td>Nurr1 overexpression enhances GDNF expression</td>
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<td>Nurr1 presence is essential for Ret expression in DA neurons</td>
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<td>Nurr1 interaction with Ret promotes neuronal migration, relocation, phosphorylation etc.</td>
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<td>GDNF promotes Nurr1-positive DA neurons from mouse mesencephalic neurospheres</td>
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**Table 1.** Interactions between cells and genes involved in our strategy. Also see Figure 1. DA, dopaminergic; GFRα1, GDNF family receptor alpha 1; SOD, superoxide dismutase; NO, nitric oxide.

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<th>GDNF-GPX-1</th>
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<td>GDNF enhances GPX-1 activity, and active GPX-1 potentiates protective properties of GDNF by inhibiting oxidative stress</td>
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**Figure 1.** Interactions among three cell types of microglia, astrocytes and DA neurons, and three effector molecules GDNF, Nurr1 and GPX-1. Our hypothesis is based on the overexpression and secretion of GDNF by astrocytes (box A), Nurr1 by microglia (box B) and overexpression of GPX-1 by DA neurons (box C). Gene-to-cell and gene-to-gene interactions are shown by simple arrows and described in Table 1 according to their arrow numbers. The overall outcome will be DA neurons highly protected against oxidative and inflammatory damages or parkinsonian toxicity (box D).
Figure 2. A schematic representation of our hypothetic strategy in vitro. Astrocytes and microglia will be transduced with lentivirus vectors carrying, respectively, GDNF and Nurr1. The transduced cells will be mixed and will be co-cultured with dopaminergic neurons transduced with GPX-1 constructs. The protective effects of each expressed factor or their combination on neurons against mediators of neuro-inflammation as well as 6-OHDA toxicity will be examined. As for Nurr1 activity, a control group will receive LPS (See text).
Figure 3. A schematic representation of our hypotetic strategy in vivo. The transduced microglia/astrocytes will be injected into the ST region of rats lesioned by parkinsonian neurotoxins before and/or after receiving cells. Lenti-GPX-1 viruses will be injected into the SN region. A control group of animals will receive LPS to induce inflammation in their ST region. The impact of the protecting factors on animals’ biochemical and behavioral symptoms will then be examined.
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