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Antioxidant Enzyme Gene Delivery To Protect From HIV-1 Gp120-Induced Neuronal Apoptosis

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Summary

Human immunodeficiency virus-1 (HIV-1) infection in the central nervous system (CNS) may lead to neuronal loss and progressively deteriorating CNS function: HIV-1 gene products, especially gp120, induce free radical-mediated apoptosis. Reactive oxygen species (ROS), are among the potential mediators of these effects. Neurons readily form ROS after gp120 exposure, and so might be protected from ROS-mediated injury by antioxidant enzymes such as Cu/Zn-superoxide dismutase (SOD1) and/or glutathione peroxidase (GPx1). Both enzymes detoxify oxygen free radicals. Because they are highly efficient gene delivery vehicles for neurons, recombinant SV40-derived vectors were used for these studies. Cultured mature neurons derived from NT2 cells and primary fetal neurons were transduced with rSV40 vectors carrying human SOD1 and/or GPx1 cDNAs, then exposed to gp120. Apoptosis was measured by TUNEL assay. Transduction efficiency of both neuron populations was >95%, as assayed by immunostaining. Transgene expression was also ascertained by Western blotting and direct assays of enzyme activity. Gp120 induced apoptosis in a high percentage of unprotected NT2-N. Transduction with SV(SOD1) and SV(GPx1) before gp120 challenge reduced neuronal apoptosis by >90%. Even greater protection was seen in cells treated with both vectors in sequence. Given singly or in combination, they protect neuronal cells from HIV-1-gp120 induced apoptosis. We tested whether rSV40s can deliver antioxidant enzymes to the CNS *in vivo*: intracerebral injection of SV(SOD1) or SV(GPx1) into the caudate putamen of rat brain yielded excellent transgene expression in neurons. *In vivo* transduction using SV(SOD1) also protected neurons from subsequent gp120-induced apoptosis after injection of both into the caudate putamen of rat brain. Thus, SOD1 and GPx1 can be delivered by SV40 vectors *in vitro* or *in vivo*. This approach may merit consideration for therapies in HIV-1 induced encephalopathy.

Keywords: superoxide dismutase, glutathione peroxidase, SV40

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Introduction:

HIV encephalopathy covers a range of HIV-related CNS dysfunction. The most severe is HIV-associated Dementia (HAD) [1]. The prevalence of HAD was estimated to be as high as 30% in patients with advanced AIDS [2], but HAD has become less common since the introduction of Highly Active Antiretroviral Therapy (HAART), [3,4]. This reduction probably reflects better control of HIV in the periphery, since antiretroviral drugs penetrate the CNS poorly. A less fulminant form of HIV-related neurological dysfunction, minor cognitive/motor disorder (MCMD), has become more prevalent and remains a significant independent risk factor for AIDS mortality [5,6].

Soluble gp120 can induce apoptosis in a wide variety of cells including lymphocytes [7], cardiomyocytes [8] and neurons [9-11]. In the brain, HIV mainly replicates in microglial cells and minimally, if at all, in neurons [12-14]. At high concentrations, HIV gp120 may be directly neurotoxic [15]. That gp120 induces apoptosis has been shown by studies in cortical cell cultures, in rat hippocampal slices and by intracerebral injections *in vivo* [16]. HIV gp120 binds neuron cell membrane coreceptors (CCR3, CCR5 and CXCR4) and elicits apoptosis, apparently via G-protein coupled pathways [17-20]. Soluble gp120 also increases glial cell release of arachidonate, which impairs neuron and astrocyte reuptake of glutamate [21] causing prolonged activation of *N*-methyl-D-aspartate (NMDA) receptor which disrupts cellular Ca^{2+} homeostasis and leads to neuron cell death [22-24]. In addition NMDA receptor activation induces formation of inducible nitric oxide synthase (iNOS). Proinflammatory cytokines, TNF-α and TGF-β, also upregulate

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iNOS [25,26]. Superoxide anion (O_2^-), a byproduct of electron transport is produced by myeloid and monocytic cells upon HIV-1 infection [27]. Superoxide dismutase (SOD) scavenges O_2^- and converts it to peroxide (H_2O_2) [28]. Peroxide is then further detoxified by catalase or glutathione peroxidase. The later oxidizes glutathione.

$$2H^+ + O_2^- \xrightarrow{SOD1} O_2 + H_2O_2 \xrightarrow{GPx1} 2H_2O$$

HIV-infected individuals have impaired antioxidant defenses [29]. Poor SOD activity leads to elevated levels of reactive oxygen species (ROS), [30] which in turn leads to depletion of intracellular antioxidants such as glutathione (GSH) [31]. Cellular vulnerability to oxidant-related injury is made worse by the fact that nitric oxide (NO) generated by NMDA-R-activated iNOS readily binds superoxide anion to form the highly reactive species, peroxynitrite (ONOO) (O_2^- + NO $\rightarrow$ ONOO) [21,32-34]. Peroxynitrite reacts with, and damages lipids, proteins and DNA [35,36].

Here we describe protection of central nervous system neurons from HIV-1 gp120 oxidant-related toxicity by gene delivery of anti-oxidant enzymes SOD1 and GPx1 using recombinant SV-40 derived vectors. These vectors readily transduce neurons *in vitro* and *in vivo*, and have been effective in studying gene transfer as protection from HIV-1 in experimental systems [37-42]. We found that rSV40 gene delivery of transgenes SOD1 and GPx1, singly or in combination, significantly mitigated neuronal apoptosis mediated by gp120. The effectiveness and degree of protection of these vectors in delivering these antioxidant enzymes was also verified *in vivo*. Antioxidant gene delivery may thus be a future therapeutic adjunct in treating CNS AIDS.

Results:

Characterization of Neurons:

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Neurons were prepared from NT-2 cells (NT2-N) as described in Methods, by differentiation using retinoic acid and mitotic inhibitors. NT2-N could be seen with refractile small cell bodies and were interconnected with neuritic processes. The cells grew on a feeder layer of large adherent flat cells, which represent immature neurons (Fig. 1a). The differentiated mature neurons were further characterized by immunohistochemical and Western analyses using antibodies vs. the neuron-specific markers, MAP-2 and Neu-N (Fig. 1b). These NT-2-N were positive for MAP-2 and NeuN, whereas undifferentiated NT-2 cells were not. Western analysis demonstrated predominant protein bands at 280 and 70 kDa (High and low molecular weight isoforms) for MAP-2, and at 50 kDa for Neu-N (Fig. 1c). Again, undifferentiated NT-2 cells were negative for both.

Dose response of gp120 in neurons:

The optimal concentration of HIV-1 Ba-L gp120 to elicit apoptosis in neurons was determined using NT2-N. Cells were incubated with 0 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml of recombinant soluble gp120 (Figs. 2a and 2b). Apoptotic bodies were analyzed using terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL). The intensity and frequency of TUNEL+ cells increased with increasing gp120 dose, up to 100 ng/ml. Higher concentrations of gp120 caused cells to detach and so were not further studied (data not shown). Thus 100 ng/ml of gp120 was used in all subsequent studies.

Effectiveness of recombinant SV(SOD1) and SV(GPx1) in transducing NT2-N cells:

Terminally differentiated NT2-N were transduced with MOI 10 of SV(SOD1) and SV(GPx1) on day 0, then again at MOI of 3 on days 3 and 5 , for a cumulative MOI of 16. Alternatively cells

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were transduced on the same schedule but with MOI=100, then 30 and again 30. For simplicity 10-3-3 regimen is referred to as MOI=10 and the latter regimen is referred to as MOI=100. The transduced neurons were analyzed for transgene expression after two weeks. Western analysis showed a predominant band at 22 kDa for SOD1 (Fig. 3a) and 24 kDa for GPx1 (Fig. 3b) respectively, both of which were more abundant in transduced, as opposed to control, groups. Transduction at 100 MOI did not increase expression beyond what was observed with 10 MOI. The activity of enzymes delivered by these vectors was studied using kinetic assays for SOD1 and GPx1. There is a low background level of enzyme activity, since SOD1 and GPx1 are normal cellular enzymes. However transduced cultures showed markedly and significantly increased activities of their respective enzymes, compared to control cultures (Figures 3c and 3d). Again, increasing transduction MOI from 10 to 100 did not further increase enzyme activity. Therefore in all subsequent experiments, transduction was done at MOI = 10.

Protection of NT2-N from gp120 apoptosis by transduction with SV(SOD1) and SV(GPx1):

Having established that 100 ng/ml gp120 produced maximal apoptosis in cultured neurons, we tested whether delivery of antioxidant enzymes SOD1 or GPx1, singly or in combination, would protect NT2-N from gp120-induced programmed cell death. Control NT2-N were also transduced with SV(HBS) and were challenged with gp120 [100 ng/ml (0.9 nM)]. Apoptosis was scored by counting total number of TUNEL positive cells in six different 10x fields. In control cultures mock or SV(HBS)-transduced cells, this resulted in 90-100 % apoptosis. NT2-N transduced with SV(SOD1) and/or SV(GPx1) expressed substantially more of the antioxidant enzymes than did control cells, as analyzed by immunostaining and western blotting (Fig.4) and were 80% protected from apoptosis ($p<0.005$) as compared to mock transduced neurons

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(Fig 4). The background level of apoptosis in these cells was less than 2-5% and was comparable among all experimental and control groups. (data not shown). The fluorescence intensity in the TUNEL assay was measured using IP lab software. Following gp120 exposure: for SV(HBS) transduced NT2-N, it was 9897 ± 124 (arbitrary units); for SV(SOD1)-treated NT2-N, it was 1565 ± 57; for SV(GPx1)-treated NT2-N, it was 1032 ± 43. Differences between antioxidant-treated and control cultures were highly significant ($p<0.005$).

When very low MOI (1,0.3,0.3) were used to transduce the neurons, less protection was observed (data not shown). Combination transduction with both viruses (SOD1 and GPx1), provided ~90% protection from gp120-induced apoptosis, which was slightly, but not significantly, better than either alone.

SV(SOD1) and SV(GPx1) protect primary fetal neurons from gp120 apoptosis:

Having established that gp120-induced apoptosis could be prevented by transduction with SV(SOD1) and SV(GPx1) in cultured neurons derived from NT-2 cells, we tested whether primary human neurons responded similarly. That is, would transduction of primary neurons with SV(SOD1) or SV(GPx1) protect them from apoptosis induced by gp120? The cell populations used for the present studies were prepared from human fetal brain as described in Methods and were >98% neurons, as indicated by positive staining for MAP-2 (Fig. 5a). Transduction efficiency was comparable to NT2-N when assayed by immunostaining: >90% of these neurons were efficiently transduced with the vectors, either alone or in combination (data not shown). Test and cultured-transduced cells were then treated with recombinant gp120 and apoptosis was measured by TUNEL assay. Transduction with SV(SOD1) and/or SV(GPx1) largely (>80%) and significantly ($p<0.005$) protected primary neurons from gp120-

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induced apoptosis (Fig. 5b) as compared to neurons transduced with the control vector, SV(HBS). Combination transduction with SV(SOD1) plus SV(GPx1) provided protection from gp120-induced apoptosis comparable to that afforded by either antioxidant transgene singly. As with NT2-N, levels of apoptosis in transduced and control cultures that were not challenged with gp120 were comparable (data not shown).

Transgene expression and protection from gp120-induced apoptosis after injection of SV(SOD1)/SV(GPx1) *in vivo*:

We then asked whether SV(SOD1) and SV(GPx1) were capable of delivering their antioxidant enzymes to brain cells *in vivo.* SV(SOD1) and SV(GPx1) were injected stereotaxically into the rat caudate putamen. The distribution of transgene expression was studied by immunocytochemistry (Fig.6) and Western analysis (Fig. 7a and 7b). Numerous transgene-positive cells were observed one week after injection of SV(SOD1) (Fig. 6). The number of cells expressing the transgenes detectably was somewhat higher when tested 14 days after vector injection as compared to 7-days (Fig. 7) When analyzed by Western blotting, high levels of transgene expression was detected both 7 and 14 days post-injection. Levels detected at the later time point were somewhat higher than at 7 days, possibly because the increased time elapsed allowed for greater protein accumulation (Fig. 7a and 7b). Very few or no cells on the uninjected side were immunopositive for the transgenes. Also rats that were mock-transduced, or transduced with SV(BugT), showed minimal immunopositivity for SOD1. Most cells that were transduced *in vivo* were neurons, as shown by immunostaining with Neuro Trace (Nissl) which was used as marker for neurons (Fig. 6). Similar results were observed after injection into the CP with SV(GPx1) constructs in rat brain (Data not shown).

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To test the effectiveness of SOD1 in protecting from HIV-1 gp120 induced apoptosis, we injected 100 ng of gp120 into the CP 2 weeks following injection of SV(SOD1). Intracerebral injection SV(SOD1) provided significant protection (>80%) from gp120-induced apoptosis when measured by TUNEL as compared to control vector SV(BugT) (Fig. 7c). The majority of cells that were protected were found to be neurons (Fig. 7c and 7d).

Discussion:

Neuron apoptosis is one of the mechanisms by which HIV causes CNS injury in AIDS patients [43,44]. Many reports suggest that HIV-induced neuron injury involves free radicals, beginning with a series of reactions initiated at the cell membrane by gp120 to generate ROS culminating in neuronal apoptosis. There is no accepted therapy for HIV-induced neuron apoptosis and our approach, involving gene delivery, is unique to the best of our knowledge. Accordingly, we hypothesized that gene transfer for ROS detoxifying enzymes and other free radical scavengers may protect CNS cells from gp120-induced apoptosis. We thus asked whether HIV gp120 elicited apoptosis in neurons and, if so, whether the antioxidant enzymes SOD1 and GPx1 delivered by recombinant SV40 vectors could protect neurons.

We used two types of cells for these studies: NTera-2 cells, a human teratocarcinoma cell line that could be induced to differentiate into NT2-Neurons [45,46], and primary neurons derived from human fetal brain [47]. In both, mature neurons comprised >98% of the cultured cells as assessed by MAP-2 and Neu-N staining. These cells predominantly express HIV-coreceptors CCR5 with lesser amounts of CXCR4 and CCR [48].

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When exposed to HIV-BaL-gp120, both primary neurons and NT2-derived neurons underwent apoptosis in a dose-dependent manner, with maximal effect at 100 ng/ml gp120 (0.9 nM) This concentration of gp120 is consistent with doses of gp120 used in studies repeated by others examining HIV-1 induced neuronal apoptosis [11,15,17]. rSV40 gene delivery increased intracellular SOD1 and GPx1 and largely protected neurons from the effects of gp120. Cotransduction with SV(SOD1) and SV(GPx1) improved protection, slightly, but not statistically significantly compared to either transgene alone.

These data are in agreement with previous reports that superoxide generated as a result of HIV infection of macrophages may be scavenged by SOD1 in a dose-dependent manner and detoxified by very high concentrations of recombinant SOD1 [49]. It is also possible that transduction with SOD1 might also mitigate the effects of HIV on microglial cells, which are the primary CNS targets for HIV infection and which produce increased superoxide when exposed to HIV [49] .

The role of antioxidants has been studied in a variety of neurological disorders using several viral gene transfer vectors. Adenovirus containing Cu/Zn SOD-1 in cerebral vessels has shown to prevent cerebral blood flow autoregulation [50] and cerebral vasopasm [51] during acute stage of subarchnoid hemorrhage. Lentiviral vectors have also been used in targeting several genes involved in neurological disorders for efficient therapeutic intervention [52]. Overexpression of GPX in rat embryonic cortical neurons using adenoviral vector (Ad-GPX) increases resistance of neuronal cells to amyloid β-peptide (Aβ) mediated neurotoxicity and

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has potential implications for gene therapy of Alzheimer's disease [53]. Overexpression of GPX using lentivirus vectors in nigral dopaminergic neurons *in vivo* has also been shown to be neuroprotective in murine models of Parkinsons disease [54]. Herpes Simplex viral vectors expressing GPX inhibit release of cytochrome c and proapoptotic mediators, and protect neurons exposed to experimental stroke and necrotic insults [55-57].

Generation of ROS in response to HIV infection leads to shifts in intracellular redox balance. This causes depletion of antioxidant species such as glutathione [31], and increases propensity to undergo apoptosis [58]. ONOO can cause cell death by several mechanisms, including nitration of tyrosine residues. Nitration of neurofilaments, actin and other structural proteins may disrupt filament assembly and impair cytoskeletal stability [32]. Our preliminary studies also indicate that gp120 may lead to increased protein nitration in neurons as well (Agrawal and Strayer , unpublished)

To determine whether the effectiveness of rSV40 vectors in antioxidant gene delivery to neurons *in vitro* might also be applicable *in vivo*, we also tested SV(SOD1) and SV(GPx1) delivery of antioxidant genes directly into the brain. Efficient transgene expression in the rat brain was noted after single injection into the CP: numerous cells, mostly neurons, expressed the transgene. We have previously reported that these vectors are effective transducing agents by intracerebral injection [59]. Numbers of transgene-expressing cells may be further increased using multiple inoculations [60,61]. We also investigated neuronal apoptosis caused *in vivo* by recombinant gp120, preparatory to attempting to assess protection using these antioxidant transgene SV(SOD1).

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In vivo transduction with SV(SOD1) substantially protected neurons from gp120-induced apoptosis, compared to *in vivo* injection of the control vector SV(BugT).

This is the first study to demonstrate that neurons can be protected from gp120-induced apoptosis by antioxidant gene transfer using SV40 vectors. We used rSV40s here since they are excellent delivery vehicles for neurons, both *in vivo* and *in vitro* [59,62-64]. Because of their transduction efficiency and effectiveness in mitigating gp120-induced apoptosis, these vectors may serve as investigative tools to elucidate mechanisms of neuron damage in HIV infection of CNS. Thus, detoxification of ROS by SV(GPx1) or SV(SOD1) protects from HIV-induced apoptosis and may serve as a potential gene therapy strategy for people with HIV encephalopathy.

Materials and Methods:

Plasmids and viral expression constructs:

SOD1 and GPx1 transgenes were subcloned in to pT7[RSVLTR]. Transgene expression is controlled by the Rous Sarcoma Virus long terminal repeat (RSV-LTR) as a promoter. SV-HBS was used as negative control recombinant virus for *in vitro* experiments, which encodes Hepatitis B surface antigen [42]. SV(HBS) cannot be used for *in vivo* experiments because immunity to the expressed hepatitis surface antigen transgene leads to elimination of transduced cells in immunocompetent rats. SV(BugT) was used as negative control vector for *in vivo* studies. SV(BugT) carries the cDNA for human bilurubin-uridine 5′-diphosphate-glucuronysyl transferase (BugT), driven by two tandem SV40 early promoters (SV40-EP) [65]. Generation of recombinant SV40 viruses has been reported in detail [41]. Briefly, recombinant

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viral genomes were transfected into COS-7 cells. We use COS-7 cells as a packaging cell line because they supply all SV-40 genes needed for virus packaging. Recombinant viral stocks were prepared as cell lysates and were purified using sucrose cushion. Virus stocks were titered by Q-PCR [66].

Cell lines: COS-7 cell line was obtained from American Type Culture Collection (ATCC) and was maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% calf serum (Hyclone, Logan, UT), 2 mM L-glutamine and containing 1.5 g/l sodium bicarbonate, 4.5 g/l glucose, 1.0 mM sodium pyruvate, penicillin (200 U/ml) and streptomycin (100 μg/ml). Human N-tera2/cloneD1 (NT-2), derived from a teratocarcinoma was obtained from Stratagene (La Jolla, CA) and induced to differentiate into NT2-Neurons (NT2-N) according to manufacturer's instructions. The cells were propogated in DMEM with nutrient mixture F-12 (DMEM/F-12) supplemented with glutamine and 10% (v/v) calf serum. Differentiation to neurons was induced by adding 10 μM retinoic acid (Sigma Chemicals,MO, USA). After the first replating, the cells were treated with mitotic inhibitors: cytosine β-D-arabinofuranoside (ara C) (1 μM), uridine (1-β-D-ribofuranosyluracyl) (Urd) (10 μM) and 5-fluoro-2'-deoxyuridine) (FUDR) (10 μM) for three weeks. After further enrichment and selective trypsinization, highly enriched neurons were harvested two-three weeks later. The neurons culture obtained were >95% pure neurons and were plated on poly-D-Lysine (Sigma Chemicals) and matrigel coated (BD Biosciences, Bedford, MA) 4-chamber slides or 24 well tissue culture plates. The neurons were characterized using MAP-2 and Neu-N antibodies by immunostaining (Chemicon International Inc., Temecula, CA) [45].

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Primary human fetal Neuronal cells: Neurons were isolated from the fetal brain as described previously [47]. Briefly, the fetal brain (obtained from the Human Fetal Tissue Bank, Albert Einstein College of Medicine, Bronx, NY) was homogenized in Hanks Balanced Salt Solution (HBSS) Ca^{2+} and Mg^{2+} free containing 0.05% Trypsin and 100 U of DNAse. Mixed brain cultures were passed through 170 μm Nylon mesh, then plated in Poly-D-Lysine coated 24 well Plates or 4-chamber slides. Non-adherent cells were removed by washing with DMEM/F-12 (Invitrogen, CA). Adherent neuronal cultures were treated with cytosine arabinoside (ara C -1 μM) for two weeks. The enriched neurons were subsequently immunostained with MAP-2 antibody.

Animals: Female Sprague-Dawley rats (200-250g) were purchased from Charles River Laboratories (Wilmington, MA). Protocols for injecting, and sacrificing animals were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee, and were consistent with AAALAC standards.

Transduction and challenge studies *in vitro*: COS-7 cells, NT-2 precursors or NT2-neurons were transduced with SV40-derived viruses SV(SOD1) and SV(GPx1) either singly or in combination on days 0, 3 and 5 with MOI of 10, 3 and 3 respectively. The cells were tested for transgene expression by Western analysis and immunostaining. The cells were maintained for 5 days in DMEM supplemented with 2% fetal bovine serum. The transduced and mock-trasnduced cells showed the same degree of viability (>95%). Recombinant HIV-1-Ba-L-gp120 (AIDS Reagent Program,NIH) (100 ng/ml)(0.9 nM) was then added to the cultures for two days. The cells were washed and cultured for another 3 days before performing TUNEL assay

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(Roche, Indianapolis, IN). The mean sum fluorescence intensity of TUNEL positive cells was calculated using IP Lab software and analyzed on fluorescence imaging/microscope (Olympus IX70).

***In vivo* transduction studies:** The rats were anesthetized with isofluorane UPS gas (BaxterHealthcare Corp., Deerfield, IL) (1.0 unit isofluorane/1.5 liters O_2 per minute) and placed in a stereotaxic apparatus (Stoelting Corp., Wood Dale, IL) for cranial surgery. Body temperature was monitored throughout the surgical procedure and maintained at 37°C by using a feedback-controlled heating device (Harvard Apparatus, Boston, MA). Glass micropipettes (1.2 •m outer diameter; World Precisions Instruments, Inc., Sarasota, FL) with tip diameters of 15 µm were backfilled with 10 µl of viral vector; SV(SOD1), SV(GPx1) or SV(BugT) which contains approximately 10^7 particles. 100 ng of gp120 was also injected using micropipettes in to the caudate putamen two weeks post transduction. The vector/gp120-filled micropipettes were placed into the caudate putamen (CP) using coordinates obtained from the rat brain atlas. For the injection into the CP, the burr hole was placed +0.48 mm anterior to bregma and -3.0 mm lateral to the sagittal suture. Once centered, the micropipette was placed 6.0 mm ventral from the top of the brain. The SV40 vectors were administered by a Picospritzer II (General Valve Corp., Fairfield, NJ) pulse of compressed N2 duration 10 ms at 20 psi until the 1 or 10 µl was completely ejected from the pipette. Following the surgery, animals were housed individually with free access to water and food. After a survival period of 7 or 14 days, animals were anesthetized via intraperitoneal injection of sodium pentobarbital (Abbott Laboratories, North Chicago, IL) (at 60 mg/kg) and perfused transcardially through the ascending aorta with 10 ml heparanized saline and 1000 ml of 4%

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paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1M phosphate buffer (pH 7.4). The animals were analyzed for apoptosis post 24 hrs after injecting gp120 9100 ng). Immediately following perfusion-fixation, the rat brains were dissected out, placed in 4% paraformaldehyde for 24 h, then in a 30% sucrose solution for 24 h, then frozen in methyl butane cooled in liquid nitrogen. The samples were cut on a cryostat (10 μm sections). General morphology of the brain was assessed by neutral red (NR) staining performed on cryostat sections. Transduction was assessed for each injected brain CP injections by serial cryo-sectioning (10 μm thick coronal sections) and immunostaining of every 10th section for SOD1 or GPx1. For each rat, the numbers of transduced or apoptotic cells in one hemisphere were counted and summed. The longitudinal diffusion of the transgene away from the injection site was assessed as the distance comprised between the most distant sections anterior and posterior to the injection site demonstrating transgene expression. General morphology of the brain was assessed by neutral red (NR) staining performed on cryostat sections.

Immunostaining: Cells were grown either on 24 well plates or 4-chamber slides treated with poly-D-Lysine and matrigel. At the indicated times post-transduction, cells were fixed with 1% paraformaldehyde for 30 min on ice, permeabilized with 0.1% Triton X-100 in sodium citrate buffer. Nonnspecific binding was blocked by treating cells with normal serum from the animal species in which the secondary antibody was raised, then immunostained with either anti-SOD1 (Stressgen,Victoria, B.C., Canada) (1:100) or anti-GPx1 (Stressgen) (1:50) and anti-MAP-2 (1:50), anti-Neu-N(1:100) (Molecular Probes Invitrogen , Carlsbad, CA) for 1 hr on ice. After extensive washes in PBS containing 1% BSA, secondaries antibodies conjugated with Alexa

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Fluor 488 (Molecular Probes, Oregon, CA) or TRITC (Sigma Chemicals) were added. The cells were washed and analyzed on fluorescence imaging/microscope (Olympus IX70).

Immunocytochemistry *in vivo*: For immunofluorescence, the coronal cryostat sections (10 µm thick) were processed for immunocytochemistry with an indirect immunofluorescence technique. After redydradation in 0.1 M PBS, pH 7.2, sections were permeabilized in PBS plus 0.1 % Triton X-100 during 10 minutes, washed twice for 5 minutes in PBS, then stained by Neuro Trace (1:100) for 20 minutes at room temperature. The sections were washed in PBS plus 0.1 % Triton X-100 then two times with PBS, then let stand for 2 hours at room temperature in PBS. Combination NeuroTrace- antibody staining, was performed using (anti-SOD1) staining first followed by staining with the fluorescent Nissl stain. Incubation with primary antibody was performed for 1 h and followed by incubation for 1 h with a secondary antibody diluted 1/100. Each incubation was followed by extensive washing with PBS. In order to stain the nuclei, the mounting medium contained DAPI (Vector Laboratories, Burlingame, CA). Specimens were finally examined under a Leica DMRBE microscope (Leica Microsystems, Wetzlar, Germany). Negative controls consisted of preincubation with PBS and 0.1% BSA, substitution of non-immune isotype-matched control antibody for primary antibody, and/or omission of the primary antibody.

Western analysis: Cells were grown on either 24 well plates or 4-chamber slides treated with poly-D-Lysine and matrigel. At the indicated times post transduction, the mock-transduced cells were harvested by trypsinization and counted. The cells were lyzed by lysis buffer containing approtonin and Phenyl Methyl Sulfonyl Fluoride (PMSF). For *in vivo* protein

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analysis, brain sections were homogenized in buffer containing protease inhibitors and DNAse and cell lysates were prepared. Total protein was estimated using BCA protein assay kit (Pierce, Rockford, IL). Total cell proteins (100 μg) were loaded in each well, then transferred to Immobilon-P Poly Vinylidine difluoride (PVDF) membrane (Millipore, Inc. USA). Equal lane loading was assesed in the using anti-glyceraldehyde-3-Phosphate-Dehydrogenase (GAPDH) antibody (1:5000). The blot was executed according to manufacturer's instructions (Roche). Anti-SOD1 and anti-GPx1 were used at dilutions of 1:500 and 1:100, respectively and anti-mouse/anti-rabbit Horseradish peroxidase (HRP) was used at a dilution of 1:5000 before addition of chemiluminiscence substrate (Roche).

Kinetic assays for SOD1 and GPx1:

SOD1 activity:

SOD1 activity was measured using SOD1 kit (R&D Systems, MN, USA) according to manufacturer's instructions. Superoxide ions (O_2^-) were generated by the action of xanthine oxidase (XOD) on xanthine. O_2^- ions convert NBT to NBT-diformazan, which absorbs light at 560 nm. Briefly, cell lysates were assayed for their ability to inhibit NBT-diformazan , which was converted to relative SOD activity. A standard curve for SOD1 was also generated in a range from 1 U to 10 U activity points. The rate of increase in absorbance units (A) per minute was calculated as:

$$\frac{\text{A560 nm at 5:30} - \text{A560 nm at 0:30}}{\text{5 Minutes}} = \text{D A560nm/minute}$$

The % Inhibition for the test sample was calculated as;

$$\frac{[(\text{D A560nm/minute}) (\text{Negative control}) - (\text{D A560nm/minute})\text{test}]}{} \times 100 = \%\text{inhibition}$$

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(D A560nm/minute) (Negative control)

SOD activity was expressed as U/L from the SOD standard activity points. Unit of SOD was defined as amount of SOD which inhibits the rate of increase in absorbance due to NBT-diformazan formation by 50%.

GPx1 activity: GPx1 assay was performed following the manufacturer's instructions using a total GPx1 assay kit (Zeptometrix, NY, USA). Here, cumene hydroperoxide was used as the peroxide substrate (ROOH). Glutathione reductase (GSSG-R) and (β-Nicotinamide Adenine Dinucleotide Phosphate) NADPH were included in the reaction mixture. The change in A340 due to NADPH oxidation was monitored which indicated GPx1 activity. Since all other reagents were present in excess, the amount of GPx1 was the rate limiting factor. The change in A340nm was monitored for 1 min, after a 40 s lag period.

Net Rate A 340nm/min= (Sample Rate A340nm/min) – (Blank Rate A340nm/min)

GPx1 activity was calculated as:

GPx1 activity (U/L) = [A340nm/min/E] x d x TV/SV

Abbreviations:

E, extinction coefficient for NADPH (0.00622 μM^{-1} cm^{-1} at 340 nm)

d, cuvette path length

TV, Total volume

SV, Sample Volume

Statistical Analysis:

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Statistical analysis was performed using using Student's paired t-test for comparison between different groups.

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Titles and Legends to Figures:

Figure 1: Characterization of neurons derived from NT-2 precursor cells. Morphology of undifferentiated (NT-2) and differentiated neurons (NT2-N) (a). The cells were treated for 6-weeks with 10 μM retinoic acid, then with mitotic inhibitors, (5-fluoro-2'-deoxyuridine, uridine, (1-β-D-ribofuranosyluracyl) and cytosine-β-D-arabinofuranoside were added for 3 weeks. The differentiated neurons were further characterized using imunofluorescence (b) where NT2 progenitors and NT2-neurons were stained with antibodies vs the neuron-specific markers, MAP-2 and Neu-N. Immunostaining with an isotype-matched immunoglobulin is shown as a negative control. Western analysis (c) was also performed for MAP-2 and Neu-N using GAPDH was used as an internal loading control.

Figure 2: Apoptosis induced by HIV-gp120-Ba-L and dose response analysis with NT2-N. Recombinant gp120 was added to neuronal cultures for two days at different concentrations from 0.1 ng/ml to 100 ng/ml. The cells were washed and cultured for another 3 days before TUNEL assay. Panel (a) shows the phase contrast and corresponding MAP-2 and TUNEL fluroscence pictures. Panel (b) represents the average of total number of TUNEL positive cells as counted in six different independent fields per experiment, for 3 different independent experiments.

Figure 3: Western and kinetic analysis of SOD1 and GPx1. Western analysis of transduced differentiated neurons is shown in Fig 4a and 4b for SV(SOD1) and SV(GPx1) respectively. Transduction using both SV(SOD1) and SV(GPx1) virus constructs yielded a predominant band at 22 kDa for SOD1 and 24 kDa band for GPx1. GAPDH was used as an internal loading

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control. For kinetic analysis NT-2N were transduced with SV(SOD1) and SV(GPx1) viruses on days 0, 3 and 5 with MOI 10,3,3 respectively or MOI of 100,30,30. They were analyzed for activities of SOD1(4c) and GPx1(4d) 10 days later, using kinetic assays. Kinetic analysis was done using SOD1 and GPx1 kits according to manufacturer instructutions on Genesys 2 spectrophotometer (see Materials and Methods).

Figure 4: Neuroprotection by rSV40-delivered SOD1 and GPx1. Cells were transduced with SV(SOD1) and SV(GPx1) at MOI 10, as described in Methods. Expression of SOD1 and GPx1 in SV(HBS)-transduced and SV(SOD1)- or SV(GPx1)-transduced post-mitotic neurons as detected by immunostaining is shown in panels (a). Both transgene expression and TUNEL fluorescence intensity were quantified by image analysis software (IP Labs). SOD1 expression in SV(SOD1)-transduced cultures was 22400 ± 250 (arbitrary units), while in SV(HBS) transduced cultures, SOD1 expression was 4796 ± 198. Similarly, GPx1 expression in SV(GPx1) and SV(HBS)-transduced cultures were 23131 ± 233 and 5874 ± 125 respectively. TUNEL fluorescence intensity in SV(HBS)-transduced cultures was 9897 ± 124. In SV(SOD1)-and SV(GPx1)-treated cultures, TUNEL fluorescence intensities were 1565 ± 57 and 1032 ± 43. The ability of prior transduction with SV(SOD1) and/or SV(GPx1) to protect NT2-N from gp120 apoptosis is illustrated in the TUNEL panel of (a), and was generated from 3 independent experiments in (b). At the time of assay, cells were tested for expression of transgenes by Western analysis (see insets). Inset panels show expression of SOD1 and GPx1 transgenes with single (above) and double (below) transduction. GAPDH was used as internal loading control. The cells were maintained for 5 days in DMEM supplemented with 2% fetal bovine serum

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before addition of recombinant gp120 [100 ng/ml (0.9 nM)] to the cultures for two days. The cells were washed and cultured for another three days before TUNEL assay.

Figure 5: Characterization and expression of transgenes SOD1 and GPx1 in human primary fetal neurons. Neurons were prepared from 14-16 week human fetal brain as described in Materials and Methods. (a) neuronal staining with MAP-2 (green) and DAPI (blue). (b) protection of primary neurons from gp120-mediated apoptosis. Primary neurons were transduced with SV(SOD1) and SV(GPx1) at MOI=10, as described in Methods or mock transduced. They were exposed to gp120 according to the same protocol we used for NT2-N. Apoptosis was analyzed using TUNEL assay. Results represent the averages of three independent experiments.

Figure 6: Expression of SOD1 following injection of SV(SOD1) into the caudate-putamen (CP). SV(SOD1) was injected into the CP, while the contralateral CP was untreated. Seven days (left panels or 14 days (right panels) after administering SV(SOD1) into the CP, sections were immunostained for SOD1 (shown in red) and for Nissl a neuron marker (shown in green). Basal endogenous levels of SOD1 are shown in lane marked control rat. The uninjected side of SOD1 transduced rats and a unrelated SV(BugT) vector were used as negative controls. Cells that are positive for both neurotrace and SOD1 are in the overlay micrographs. Nuclei were also stained by DAPI.

Figure 7: Western analysis of SV(SOD1), transduction of rat brain caudate putamen and protection from gp120 induced apoptosis *in vivo.* After injection with vector SV(SOD1) at day 7

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and 14 days earlier, rat brains were sectioned (uninjected and injected sides) and homogenized. (a) Western analysis of transduction with SV(SOD1). SOD1 was seen as a predominant band at 22 kDa, comparing injected and control uninjected sides at 7 and 14 days. GAPDH was used as an internal loading control. (b) Densitometric analysis of the intensity of SOD1 protein bands, correcting for GAPDH band intensity. Panel (c) represents TUNEL fluorescence (shown in red) as observed in brain of SV(SOD1) transduced rat when compared to control SV(BugT) transduction. The majority of cells that were TUNEL positive were found to be neurons (stained with neurotrace in green). Nuclei were also stained with DAPI. Total number of TUNEL positive cells were found to be significantly reduced in rats transduced with SV(SOD1) as compared to SV(BugT) (d).

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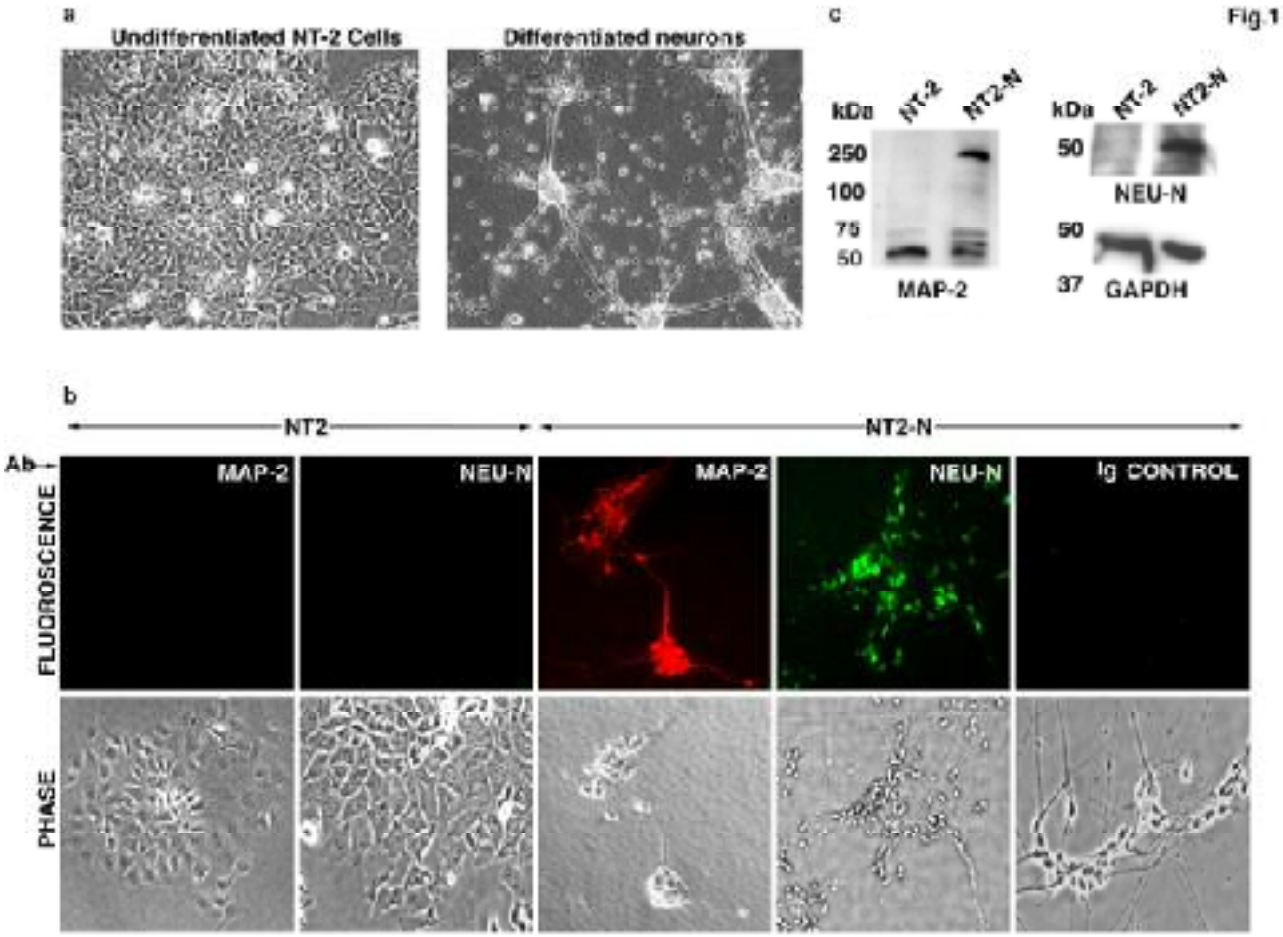

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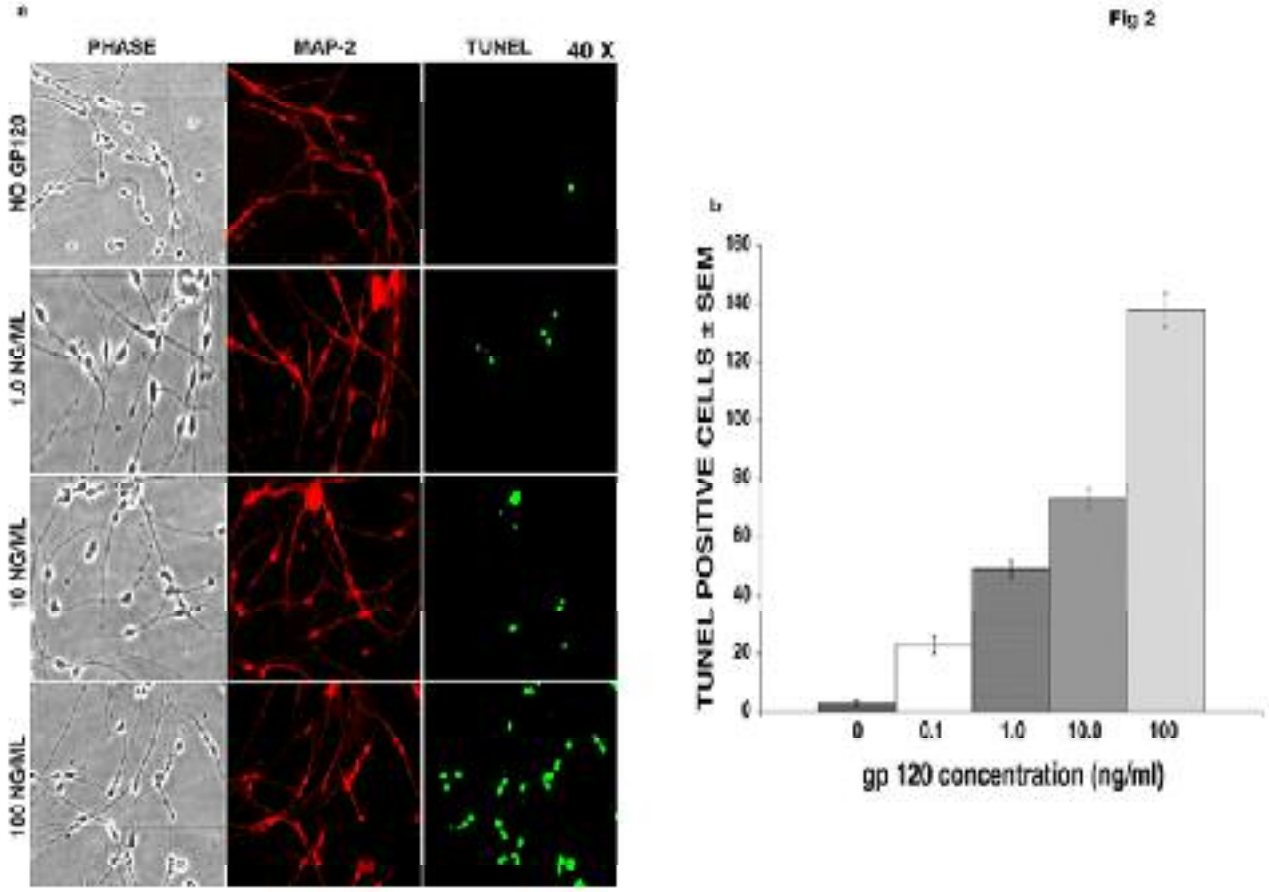

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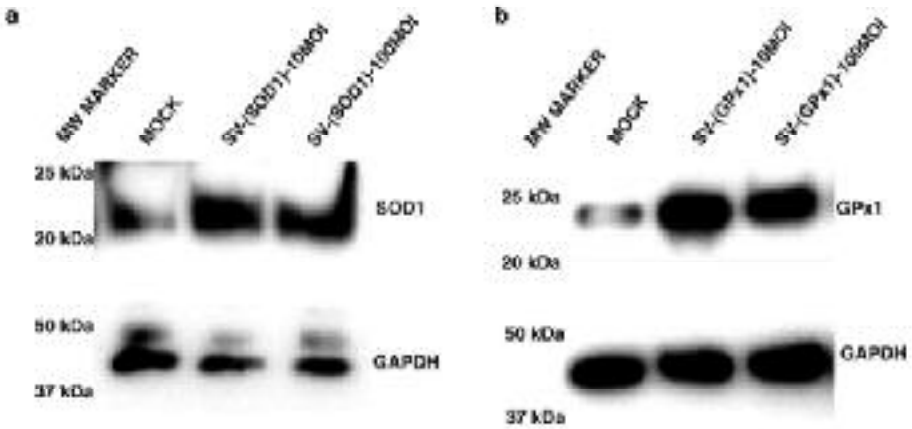
Fig.3



c

SOD1 Activity

Treatment	SOD1 activity (U/L)
MOCK	5900 ± 769
SV(SOD1)-10 MOI	*14100 ± 1112
SV(SOD1)-100 MOI	*14500 ± 598

* p<0.005 ,compared to mock

d

GPx1 activity

Treatment	GPX Activity (U/L)
MOCK	322 ± 44
SV(GPx1)-10 MOI	*759 ± 43
SV(GPx1)-100 MOI	*891 ± 15

* p<0.05,compared to mock

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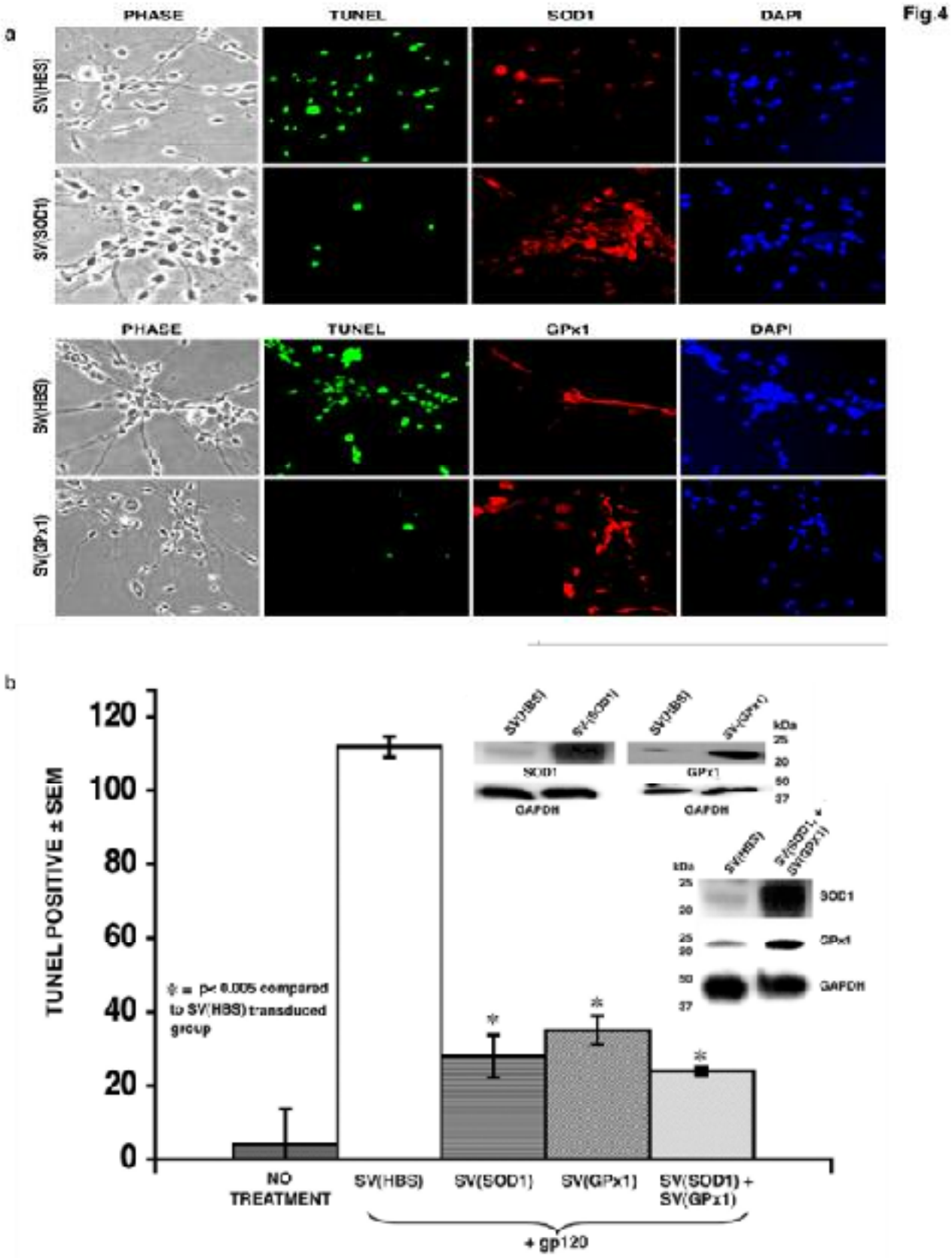

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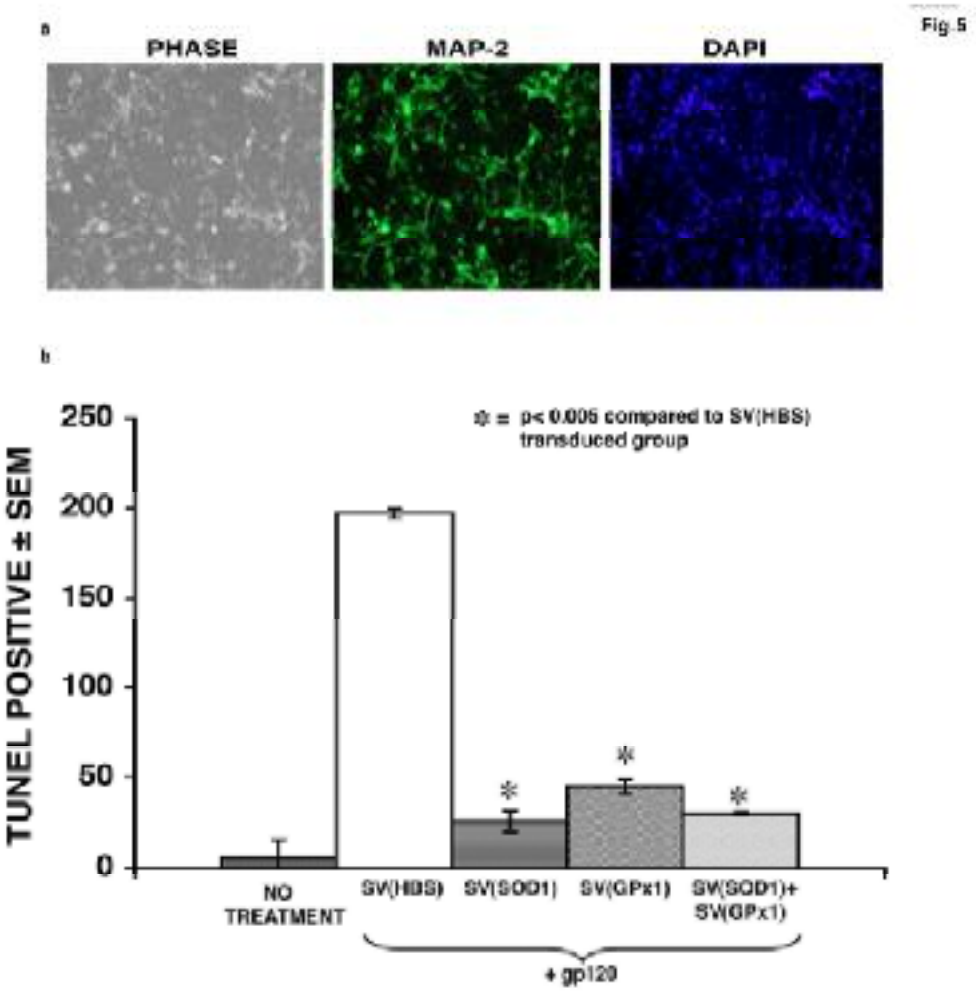

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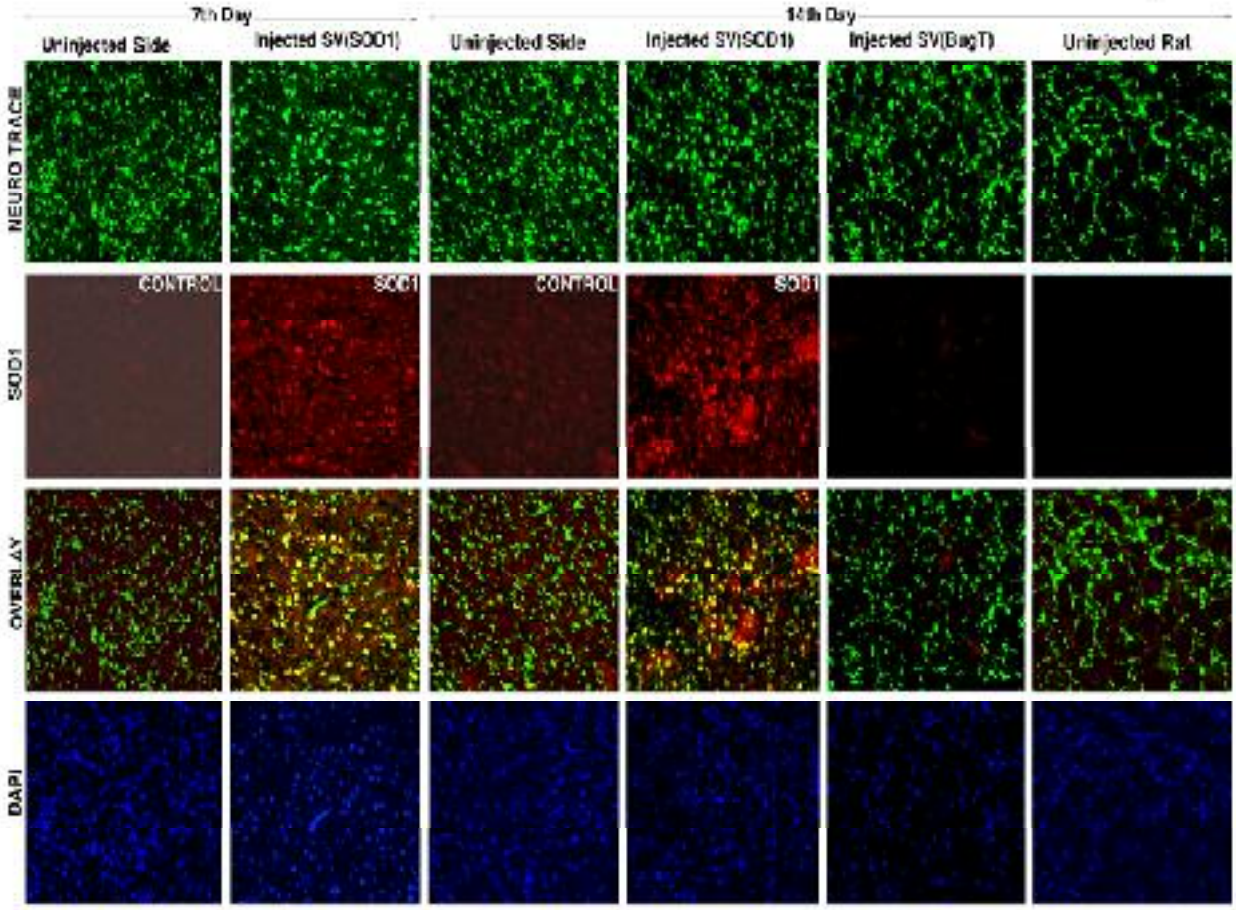
Fig 6



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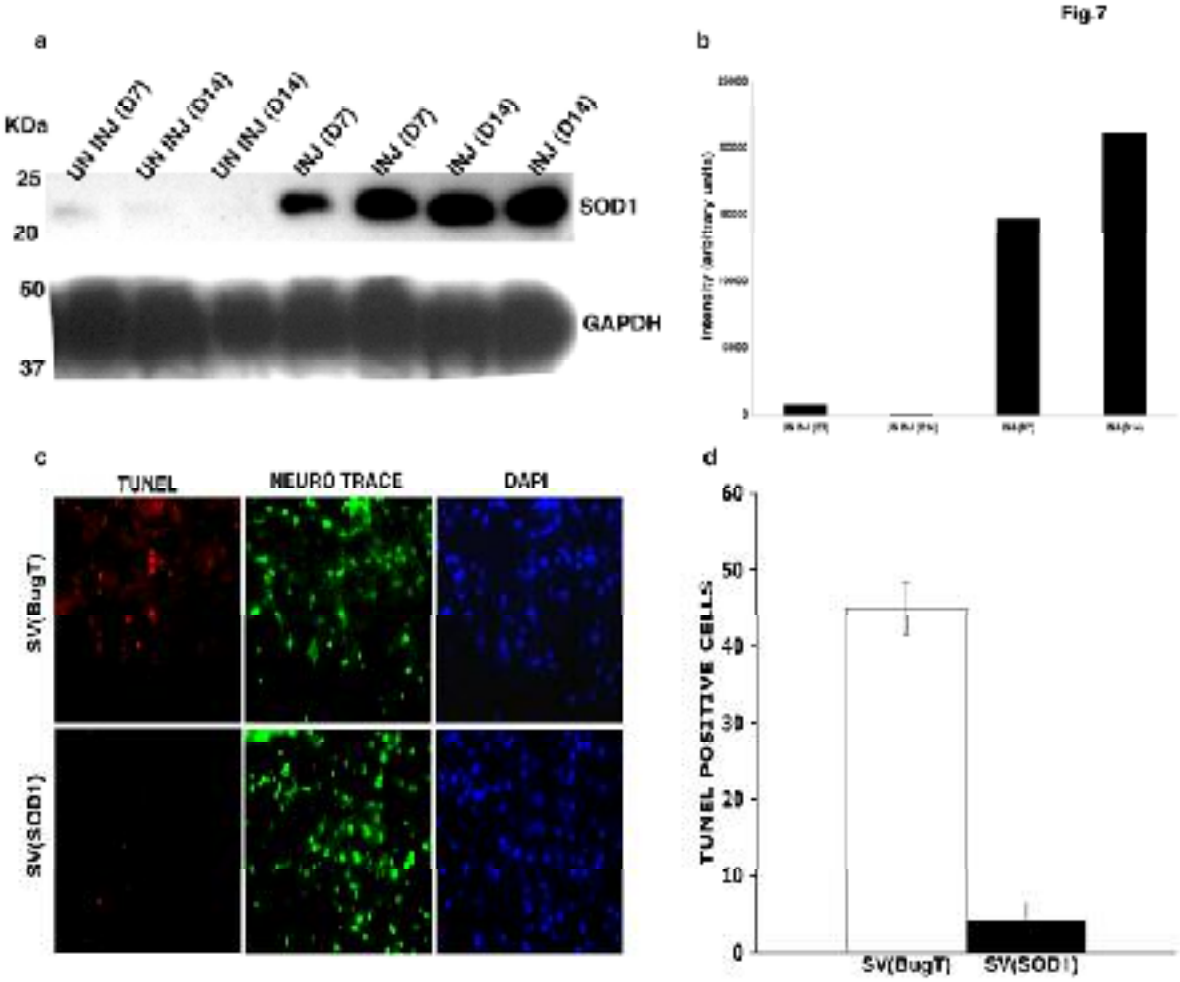

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