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TITLE: The Impact of Exercise on the Vulnerability of Dopamine Neurons to Cell Death in Animal Models of Parkinson's Disease

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14. ABSTRACT

Parkinson's disease results in part from the loss of dopamine neurons. We hypothesize that exercise reduces the vulnerability of dopamine neurons to neurotoxin exposure, whereas stress increases vulnerability. We have outlined experiments to test this hypothesis in rats treated with one of several neurotoxins, beginning with 6-hydroxydopamine. Over the past year, we increased the size and training of our research team and made a number of observations of direct relevance to our hypothesis. We also have received permission to expand our original Statement of Work to include critical studies on the mechanism of the actions of exercise, using both in vivo and in vitro approaches. Our focus continues to be on the effects of stress and exercise on the vulnerability of DA neurons, and the role played in these phenomena by trophic factors and intracellular signaling cascades.

15. SUBJECT TERMS
Basal ganglia, dopamine, ERK, exercise, MAPK, neuroprotection, Parkinson's disease, preconditioning

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Introduction:

Parkinson's disease (PD) results in part from the progressive loss of dopamine (DA) neurons projecting from substantia nigra (SN) to striatum. Although the cause of this neurodegenerative process is unknown, one candidate is oxidative stress. This is likely to result from exposure to environmental toxins, perhaps coupled with one or more bases for increased vulnerability. Such increased vulnerability could include genetic predisposition, emotional or physical stress, or exposure to certain recreational drugs. We have developed the hypothesis that exercise *reduces* the vulnerability of DA neurons to neurotoxin exposure under basal conditions and blocks stress-induced exacerbation of toxin-induced DA neuron loss.

In this many of our studies, adult male rats are given one of several exercise regimens prior to toxin exposure. Behavioral, biochemical, and histological analyses are then used to determine (1) whether the neuroprotective effects of cast-induced limb use in rats treated with 6-hydroxydopamine (6-OHDA) are also seen with other forms of exercise; (2) how much exercise is required, when must it occur, and how permanent are the effects; (3) if exercise also protects against the increased vulnerability to toxins caused by other stressors; and (4) the generality of our results with 6-OHDA to other toxins. We have recently requested and received permission to expand our Statement of Work (SOW) so that we can also include studies that explore the mechanism by which exercise and stress interacts to influence the vulnerability of DA neurons. Our progress report presents observations made both with in vivo and in vitro models. In addition to presenting our 2005-6 findings, this report includes a brief summary of in vitro observations made prior to 2005-6 in order to place the progress of the past year into some context.

Body:

1. Recruitment and training of staff

In May 2005, we submitted and had approved a revised Statement of Work (SOW). A key feature of our revised SOW was that it allowed us to pursue the cellular mechanism by which trophic factors such as GDNF are able to protect DA neurons from oxidative stress. In order to perform these experiments, we added several individuals the project. They included Anthony Liou, a highly experienced senior molecular and cellular neurobiologist who is now one of the principal investigators on this project, Lihua Li, a postdoctoral fellow with considerable experience in molecular biology, and Juliann Jaumotte, a well-trained research assistant working with cell cultures.

2. Effect of GDNF is associated with a reduction in the vulnerability of DA neurons to oxidative stress in each of our models

Many of our studies over the past 5 years have utilized ***MN9D cells*** as models of DA neurons (e.g., (Perez et al., 2002; Ugarte et al., 2003; Leak, in press). These cells have a number of the salient characteristics of DA neurons, including the ability to synthesize and store DA and to respond to GDNF. We find that 6-OHDA produces a concentration- and time-dependent death of these cells, and that these effects are attenuated by the addition of GDNF. Specifically, GDNF (10 ng/ml) protected MN9D cells against the nuclear condensation normally caused by 6-OHDA (250 μM) (Ugarte et al., 2003). Likewise, although previous studies of primary cells failed to obtain specific 6-OHDA-induced DA cell loss (Ding et al., 2003), we have developed a paradigm to obtain such selective effects on ***primary dissociated cultures*** of DA neurons from *rats* and *mice*. Thus, we prepared dissociated cultures from the SN region of rat pups (P0) and exposed them to 6-OHDA (40-250 μM; 15 min), producing a specific loss of TH^+ cells while sparing GABAergic neighbors. In this preparation we observed that GDNF (100 ng/ml) blocked 6-OHDA-induced TUNEL labeling at 3 hr (Ding et al., 2004).

3. The effects of GDNF may be due in part to activation of ERK in DA neurons

A major focus of our work is to determine the signaling cascades associated with GDNF-induced protection of DA neurons. We have found that exposure of ***MN9D*** cells to GDNF (10-100 ng/ml for 15 min) results in an increase in ERK1/2 and ERK5 *phosphorylation*, an increase in ERK *activity* as assessed in a kinase activity assay using the transcription factor Elk1, a well-known ERK target, and an increase in *pCREB*. The activation of ERK appeared to participate in the neuroprotective effects of GDNF since U0126, an inhibitor of MEK, eliminated the protective effects of GDNF (Ugarte et al., 2003).

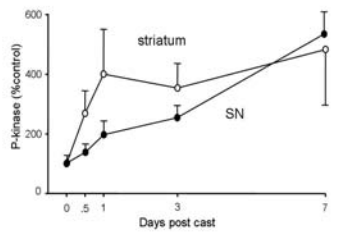

Figure 1: Effect of casting on pERK1/2 in striatum and SN as function of time after casting. Rats were sacrificed by microwave irradiation before analysis by Western blot. Shown are means ± SEM for 4-6 animals per group.

We also examined the effect of forced limb use and of GDNF on activation of ERK1/2 in our ***animal model of PD***. Western immunoblotting showed that forced use of one limb increased pERK1/2 by 4-fold in the contralateral striatum at 24 hr, and levels remained high for the 7-day casting period. In the SN, the increase in pERK1/2 was more gradual but reached a comparable level within 7 days (Figure 1). We are in the process of determining by immunocytochemistry whether the exercise-induced changes in pERK are localized to DA neurons. In the meantime, we have shown that the direct injection of GDNF into either rat and mouse striatum produced a marked increase of pERK1/2 in both striatum and SN. This occurred primarily within tyrosine hydroxylase immunoreactive (TH^+) cells of the SN, as indicated by double label confocal microscopy (see Figure 2).

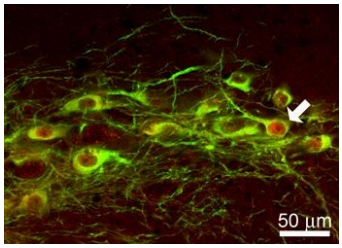

Figure 2: Effect of GDNF (4.5 µg) on pERK1/2 in mouse SN 24 hr after an intrastriatal infusion. Shown is the immunohistochemical localization of pERK and TH^+ in the SN as visualized by confocal microscopy. Individual images indicate presence of pERK both in cytoplasm and nucleus of TH cells.

4. Role of ERK5 in cell survival

ERK5 is also a member of the MAPK family. Although its biological function is poorly defined, it is known to be activated by neurotrophic factors and thus may play a role in cell survival. Indeed, our preliminary data indicate that GDNF (10 ng/ml) activates ERK5 in MN9D cells and that U0126 (10 µM), which blocks the protective effects of GDNF, also blocks ERK5 activation. We also observed that U0126 decreased basal survival of these cells. Since U0126 blocks the phosphorylation of ERK5 as well as ERK1/2, we wished to determine which ERK isoform(s) were critical to this effect. Cells were transfected with either dnERK5 or dnMEK1, the specific upstream activator of ERK1/2. Transfection of MN9D cells with either dominant negative construct reduced basal cell survival (Figure 3). Moreover, transfection of the cells so as to increase ERK1/2 activity inhibited 6-OHDA-induced cell death. A similar trend was observed for treatments that increased ERK5 activity but the effect was not significant (data not shown). These studies demonstrate a role for ERK5 in basal DA cell survival, and may also point to a difference between ERK1/2 and 5 in influencing protection of DA cells from oxidative stress (Cavanaugh, 2006, in press).

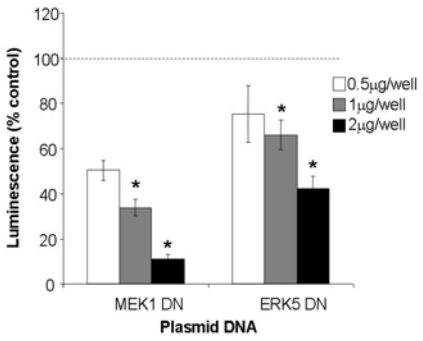

Figure 3: Effects of transfection with dominant negative MEK1 or ERK5 on basal MN9D cell survival. Cells were also co-transfected with a luciferase reporter plasmid and the number of lucerfase-positive cells counted after 24 hr. Shown is the mean ± SEM. Shown are means ± SEM. Results are representative of 3 independent experiments.

5. The effect of oxidative stress on pERK1/2 in DA cells

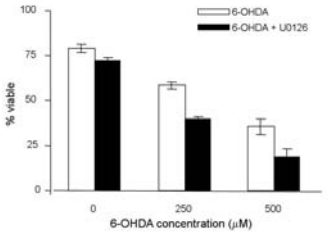

Figure 4: Effect of U0126 (5 μM) on vulnerability of MN9D cells to 6-OHDA. U0126 was added 1 hr prior to and during 6-OHDA treatment to block the first pERK1/2 peak Cell viability was measured by Hoechst stain. Data represent the mean value ± SEM of 3 independent experiments.

In our in vitro studies with ***MN9D cells*** we also observed that ERK was activated by 6-OHDA itself. This initially appeared to us to be paradoxical since it suggested that a toxic stimulus initiating a *neuroprotective* response. One possibility was that pERK served as a *pro-survival* response after GDNF and a *pro-death* response after 6-OHDA. A precedent for such a dichotomy exists. However, it was also possible that the pERK response to 6-OHDA we saw represented a defensive reaction to oxidative stress, and this appears to be the case: MN9D cells were treated with 6-OHDA (250 μM) and the time course of changes in pERK isoforms was determined. We observed that pERK1 and pERK2 were increased 25-fold at 15 min but returned to baseline by 30 min. After removing 6-OHDA, a smaller sustained peak in pERK1/2 arose between 3-6 hr and persisted for several more hr. This late pERK peak was temporally associated with activation of caspase-3 and cell death. Phosphorylation of CREB, one of the molecules that ERK phosphorylates through its action on RSK, also occurred during both the transient and sustained 6-OHDA-induced pERK activation. Addition of U0126 (5 μM) before and during toxin exposure to block the first pERK peak, significantly increased cellular vulnerability to 6-OHDA (50-250 μM) (Figure 4; Lin et al., ms submitted). No such effect was seen if U0126 was provided after this initial peak. These data suggest that the initial transient activation of ERK after oxidative stress is a compensatory response to reduce cellular vulnerability. Inhibiting the second, sustained pERK peak had no detectable effect on cell viability (Lin, 2006, submitted).

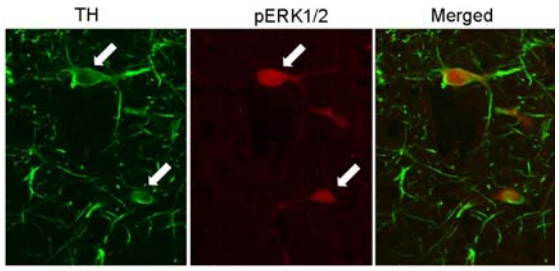

Figure 5: Endogenous pERK1/2 (red) in rat SN 24 hr after intra-MFB injection of 6-OHDA (3 μg/ 2 ul). Note absence of TH (green) in the nucleus of the two large cells in the left hand panel contrasted by the presence of pERK throughout the cell in the middle panel (arrows).

Using double labeling with Hoechst and an antibody to pERK1/2 we observed that the early activation peak was largely confined to the cytosolic compartment. Curiously, this peak remained visible at 30 min, although by Western blot it had largely receded by that time. We are in the process of trying to understand this discrepancy. In preliminary ***animal studies***, 6-OHDA (3 μg) into the MFB caused a rapid but transient increase in pERK1/2 in TH^+ cells of SN that could be detected as early as 1 hr post-infusion and then decreased over the next 11 hr (Figure 5). In this case, pERK was located in the nucleus as well as the cytoplasm. We are in the process of assessing whether this pERK response is protective or contributes to cell death after 6-OHDA.

6-OHDA generates oxidative stress as a result of forming a number of reactive molecules, including H_2O_2, and ERK1/2 is activated by H_2O_2 in dissociated cultures of ventral mesencephalic. Although this occurs in many types of cells in this culture, we could distinguish TH^+ cells exhibiting ERK activation by dual antibody labeling. The specificity of this staining was confirmed by the inclusion of U0126, a MEK-1 inhibitor, which eliminated the pERK immunostaining in all cells. Thus, with the aid of our consultant, Simon Watkins, Director of our Imaging Center, we can now quantify kinase activation within individual SN cells.

6. Development and initial utilization of molecular probes for the study of ERK in neuroprotection

Developing constitutively active (CA) forms of ERK. The capacity to over-express active ERK isoforms is essential to our research objectives. It has been shown, however, that substituting T183 and Y185 on ERK2 with glutamate does not mimic phospho-T183 and phospho-Y185. The resulting double mutant cannot function as the constitutively active version of the kinase. To circumvent this problem, we employed co-expression of caMEK with wt ERK1 or 2 respectively, and have shown that we can over-express pERK1 and pERK2 (Figure 6).

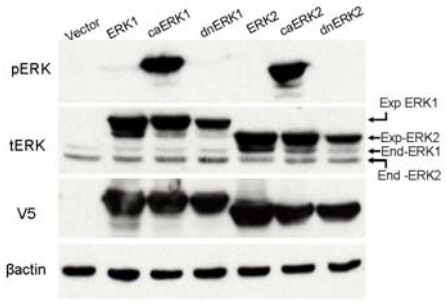

Figure 6. Ca and dnERK1 and ERK2 expression in HEK293 cells. The coding sequences of each construct was first cloned into pcDNA3.1 with V5 and His tags and transfected. Exp, expressed; End, endogenous

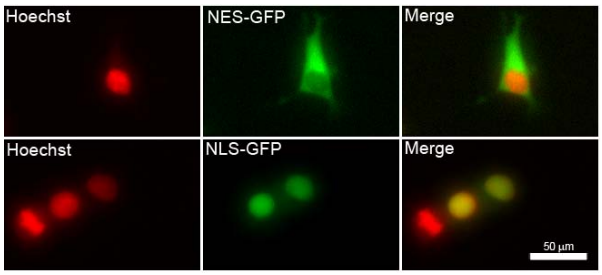

Figure 7: Localization of GFP fused with NES or NLS, and transfected into MN9D cells using Lipofectamine (Invitrogen). Nuclei were stained for Hoechst (red) and GFP was visualized by green immunofluoresence.

Directing the cellular localization of key molecules: It is important that we be able to control the localization of pERK isoforms if we are to determine conclusively whether the compartment within which pERK resides is a crucial factor in determining its impact on cell survival. As a first step in accomplishing this, we created constructs in which either the NLS or NES sequences were fused to EGFP or to ERK. We then expressed them in HEK293 cells and in MN9D cells. These sequences successfully directed the fused proteins to the nucleus (NLS) or cytoplasm (NES) (Figure 7, above). Furthermore, we have mutated the leucine residues at 33 and 37 to alanine on the caMEK1 gene to disrupt the NES motif and the aspartic acid at 316 to alanine on the ERK2 gene (termed 316A) to abrogate its association to MEK1 without affecting its capacity to be phosphorylated (activated) and function as a kinase. This construct was then co-expressed in HEK293 cells with the ERK2-316A gene fused with NLS sequence. Twenty-four hours post-transfection, the cells were immunostained with pERK and counter-stained with Hoechst staining. The wt ERK2 gene with NLS sequence was used as control. Without the NLS sequence, the pERK2 was localized largely in the cytoplasm. However, after the modification, the pERK2 in the nucleus was significantly increased, reflecting the expected function of NLS sequence. Moreover, the

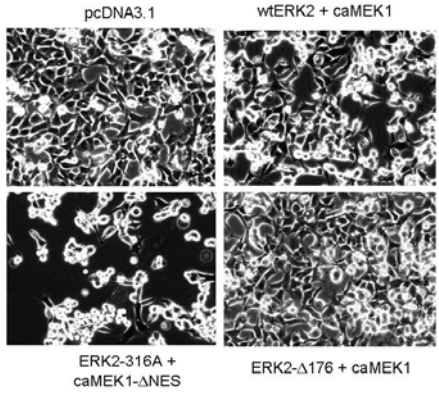

Figure 8: Effect of transfecting constructs designed to alter the location and activity of pERK on the viability of HEK293 cells as measured by a WST1 assay. The results suggest that transfection of ERK2-316A-NLS plus caMEK1-ΔNES, which increased pERK in the nucleus, resulted in a persistent nuclear expression of ERK (bottom left panel. In contrast, an ERK construct modified to remain associated with MEK (ERK2-Δ176) plus a caMEK1, thereby promoting the presence of cytoplasmic pERK, caused no loss of viability (bottom panel, right). Our viability data have been confirmed by flow cytometry.

co-expression of these two constructs resulting in a significant increase in cell death, both under basal conditions (Figure 8, previous page) and in response to H_2O_2. The corresponding NLS constructs fused to other ERK isoforms and the NES constructs for the all ERK isoforms together with the corresponding dnERK fused sequences are being constructed. We also can separate nuclear from cytoplasmic proteins by subcellular fractionation followed by Western blotting.

7. Transient ERK activation affects the up-regulation and activation of RSK

In our 6-OHDA cell model, we have shown that inhibition of the first peak of biphasic ERK activation accentuated MN9D cell death, suggesting that this transient ERK activation conferred protection against the 6-OHDA insult (Lin, 2006, submitted). We have now shown that the transient ERK activation was correlated with up-regulation and phosphorylation of RSK. These changes were abolished when the cells were pre-treated with U0126, supporting our hypothesis that protection conferred by ERK pathway is mediated in part via RSK (Figure 9).

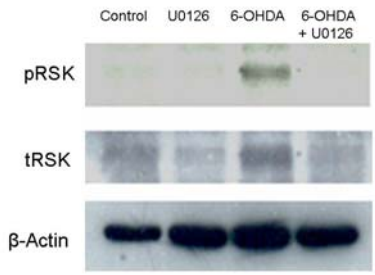

Figure 9: Western blot showing 6-OHDA-induced activation of RSK that is blocked by the pERK inhibitor U0126

8. Preconditioning of MN9D cells by 6-OHDA and methamphetamine

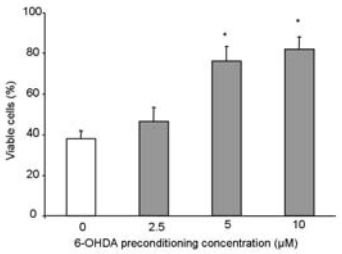

Figure 10: Effect of prior exposure of MN9D cells to subtoxic concentrations of 6-OHDA on the subsequent response to a concentration of 6-OHDA that normally represents the approximate LD_{50} (50 μM).

Our basic assumption is that protective treatments alter both post-translational and translational events so as to reduce the vulnerability of the affected cells. Such treatments include GDNF and 6-OHDA (despite its neurotoxic consequences) Given our observation that the 6-OHDA-induced phosphorylation of ERK serves to reduce the cell death caused by that toxin, it seemed reasonable to predict that low concentrations of 6-OHDA could actually precondition cells against later higher concentrations of 6-OHDA. Last year we showed that this was the case. Brief exposure to subtoxic 6-OHDA (2.5 – 10 μM) significantly reduced the vulnerability of MN9D cells to a 30 min-exposure to a toxic 6-OHDA concentration (50-200 μM) delivered 6 hr later when viability was measured 24 hr later by Hoechst staining (Figure 10) (Leak, 2006, in press).

We have gone on to show that 6-OHDA preconditioning was associated with fundamental changes in the protein profile of the MN9D cells, which included changes in gene expression, alterations in protein synthesis, and post-translational state. Although we have not yet examined the effects of GDNF on gene expression in these cells, we have performed preliminary analyses of the effects of 6-OHDA alone. MN9D cells, when exposed to 6-OHDA, show a rapid, robust change in expression profile using MG430 whole genome oligonucleotide microarrays. For example, 6 hr after a 30-min exposure to a sub-toxic concentration of 6-OHDA (5 μM) we identified a number of gene expression changes related to cellular signaling, apoptosis, oxidative phosphorylation, chaperone molecules and structural proteins. In addition, there was increased phosphorylation of ERK, Akt, and JNK, and inhibitors of the phosphorylation of each kinase also blocked preconditioning.

Key Research Accomplishments:

- Provided further training to staff; increased staff size.
- Further developed cellular models and associated molecular probes for the study of neuroprotective treatments for DA neuron cell death.
- Demonstrated an influence of several ERK isoforms in the regulation of basal viability and vulnerability to 6-OHDA in cellular models.
- Identified several of the mechanisms underlying preconditioning by 6-OHDA and methamphetamine in an in vitro model.
- Replicated our basic finding that the increased limb use was associated with contralateral casting is neuroprotective.
- Demonstrated that exercise not only reducef the behavioral and neurochemical effects of 6-OHDA in the rat but also prevented the loss of DA neurons.
- Demonstrated that the neuroprotective effects of intracerebral GDNF is associated with a delayed restoration of the DA phenotype and an activation of ERK1/2.
- Initiated a study of the impact of voluntary running on trophic factor levels and the neurotoxic effects of 6-OHDA.

Reportable Outcomes:

- Like exercise, GDNF protects DA neurons from 6-OHDA. However, the restoration of a DA phenotype requires several weeks, presumably because the neurons temporarily alter their pattern of gene expression so as to focus on combating the toxic effects of oxidative stress.
- The preconditioning associated with exposure to low levels of 6-OHDA results in part from a combination of the activation of MAP kinases and increased protein synthesis. The preconditioning associated with exposure to low levels of methamphetamine may have a somewhat different etiology and involve the activation of MAPK phosphatases.

Conclusions and Plans for Year 04:

Exercise continues to be a viable approach to the treatment of PD. Moreover, understanding its mechanism of action should provide insights into the development of drug therapies. Thus, we plan to examine the impact of environmental complexity and voluntary running on the neurotoxic effects of 6-OHDA and on the level of trophic factors in the brain. WE also will begin to examine the impact of stress on the response to 6-OHDA. A second, and complimentary, focus will be to examine in more detail the relation between the kinetics of MAPK responses to GDNF and to 6-OHDA on the one hand and cellular viability on the other. Finally, we feel that the phenomenon of preconditioning will provide further insights into the relation between mild cellular stress and the vulnerability of DA neurons and will pursue these studies using both in vivo and in vitro models.

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