AD_____

Award Number: DAMD17-03-1-0492

TITLE: Endoplasmic Reticulum Stress as a Mediator of Neurotoxin-Induced Dopamine Neuron Death

PRINCIPAL INVESTIGATOR: Robert E. Burke, M.D.

CONTRACTING ORGANIZATION: Columbia University
New York, NY 10032

REPORT DATE: July 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/07/2007	Final	01-jul-2007-30-jun-2007

4. TITLE AND SUBTITLE

Endoplasmic Reticulum Stress as a Mediator of Neurotoxin-Induced Dopamine Neuron Death

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0492

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Robert E. Burke, M.D

E-Mail: rb43@columbia.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

: Columbia University
New York, NY 10032

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Programmed cell death (PCD) is an important mediator of neural degeneration in Parkinson's disease (PD). The goal of this proposal was to examine in vivo the possible role of ER stress, a mediator of PCD, in dopamine neuron death. This was done by studying mice with targeted deletions of CHOP, an upstream transcriptional mediator, and caspase-12, a downstream mediator, of ER stress-induced apoptosis. We have found that CHOP is universally expressed in neurotoxin models of PD, and that it is an essential mediator of apoptosis in the 6OHDA neurotoxin model. The CHOP null mutation does not, however, protect dopamine neurons in the chronic MPTP model, indicating that these two models are mediated by distinct mechanisms. Although CHOP is best known as a death mediator due to ER stress, we were unable to confirm the presence of ER stress in the 6OHDA model by analysis of BiP and the XBP-1 splice variant. Furthermore, we have shown that caspase-12 null mice are not protected from 6OHDA. Since caspase-12 is a critical mediator of PCD due to ER stress, these results suggest that the upregulation of CHOP in the 6OHDA model is not mediated by ER stress, but rather oxidative insult. In the final year of this award, we have found that homozygous JNK2/3 double null mutations diminish the induction of CHOP, indicating that the MAPK pathway regulates its expression. We have found also that JNK2 and JNK3 are essential mediators of neuron death in this neurotoxin model.

15. SUBJECT TERMS apoptosis, programmed cell death, 6-hydroxydopamine, MPTP Parkinson disease

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	54	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

INTRODUCTION

There is a growing consensus that the molecular processes of programmed cell death (PCD) are important mediators of neural degeneration in Parkinson's disease (PD) and related disorders. However, while important recent advances in PD research have implicated both environmental and genetic factors in the pathogenesis of the disease, it has been unclear how these factors initiate the PCD cascade. The recent advances in our understanding of the genetic basis of PD, related to synuclein mutations which foster protein aggregation, and parkin mutations which result in a loss of functional ability to ubiquitinate difficult-to-fold proteins, have suggested a possible role for endoplasmic reticulum (ER) stress. In addition, it has been shown by analysis of gene expression in neurotoxin models in tissue culture, that ER stress may play a role in the PCD of dopamine neurons (1,2). The goals of this proposal have been to examine in living animals whether CHOP, an upstream transcriptional mediator of ER stress-induced apoptosis, and caspase-12, a downstream mediator, play a role in PCD of dopamine neurons in neurotoxin models of parkinsonism. These goals have been achieved by studying mice with null mutations for these mediators. The sensitivity of the null animals to the induction of apoptosis in dopamine neurons has been examined in well-characterized and validated models of parkinsonism: intrastriatal injection of 6-hydroxydopamine in immature and adult mice, and chronic, systemic injection of MPTP in mice. Since the original submission of this proposal, we made an important observation relevant to the molecular basis of PCD in dopamine neurons which led us to expand upon our original goals. We determined that the two kinases which phosphorylate the N-terminal of the transcription factor c-jun (JNK2 and JNK3) are essential mediators of PCD in dopamine neurons: in mice with combined JNK2/JNK3 null mutations, there is a complete abrogation of PCD induced by 6OHDA. Given our demonstration in the early work supported by this proposal that CHOP is also an essential mediator of PCD, we asked, where does CHOP lie in the molecular pathways of PCD in relation to JNK2 and JNK3? We addressed this question by examination of the expression of CHOP in mice null for JNK2, JNK3 and both JNK2/3, following 6OHDA administration.

BODY

In our original proposal, we submitted preliminary data which indicated that ER stress is likely to occur and to be a mediator of programmed cell death (PCD) in neurotoxin models of parkinsonism. Our colleague and collaborator on this proposal, Dr Lloyd Greene, had shown that the neurotoxin 6-hydroxydopamine (6OHDA) induces the expression of a number of mediators of an ER stress response in PC12 cells: ATF4, CHOP, BiP, phosphorylated PERK and others (1). A similar induction was noted on treatment of the cells with MPTP. He demonstrated that the ER stress response was likely to be mediating cell death in this culture model because sympathetic ganglion neurons derived from mice null for PERK, a mediator of a protective pathway in ER stress, were more sensitive to 6OHDA (1). Very similar findings were reported by Holtz and O'Malley for MN9D cells (2). The critical question which we therefore sought to

address in this proposal is whether ER stress occurs in these neurotoxin models *in vivo*, and if so, whether it plays a role in mediating PCD.

On the basis of these preliminary observations, we proposed three tasks to delineate the functional roles of CHOP and caspase-12, a downstream mediator of PCD in ER stress, in dopaminergic neurotoxin-induced PCD in living animal models. We proposed to do this by studying the effects of null mutations for these mediators on dopaminergic cell death induced by 6OHDA and MPTP.

***Task 1*. To determine if CHOP is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

The work for this task was completed, and published in the *Journal of Neurochemistry*. We have appended to this Final Progress Report a copy of the published manuscript. All of the work completed for this task is presented in this full manuscript.

***Task 2* To determine if CHOP expression is a general feature of neurotoxin-induced apoptosis in dopamine neurons of the substantia nigra *in vivo*.**

In relation to Task 2, we report in the manuscript that CHOP induction is a general feature specifically of neurotoxin-induced apoptosis in dopamine neurons, but it is not a general feature of all apoptosis in dopamine neurons. CHOP protein expression is observed in all of the 6OHDA and MPTP models tested, but it is not observed in apoptosis due to natural cell death, or that due to induction of natural cell death by axotomy (Figure 3 in the manuscript).

In the manuscript we also show that CHOP null mice treated with MPTP demonstrate equal levels of apoptosis as those observed in wildtype mice, and they show an equal degree of dopamine neuron loss. Thus, we have shown that there are fundamental differences in mechanisms of toxicity in the 6OHDA and MPTP models insofar as CHOP is concerned. To our knowledge, this is the first demonstration of a clear difference in mechanisms between these two models *in vivo*. This finding, however, is in keeping with the *in vitro* observations of Holtz and O'Malley, who noted a much more robust induction of CHOP, and a broader ER stress response in the 6OHDA model in comparison to the MPTP model.

Studies undertaken in response to Reviewer's comments

Our original proposal received a very fair and thorough review, and we decided that it is important to address one issue raised by the Reviewers. It was pointed out that expression of CHOP alone is not definitive evidence for the occurrence of an ER stress response. CHOP induction can occur under circumstances of oxidative stress and amino acid starvation, for example. The Reviewers therefore recommended that we examine other indicators of ER stress in our models. We selected two. One is the BiP chaperone protein, which is often (but not always) upregulated in ER stress. The other

is the ER stress splice variant of the transcription factor XBP-1, which is generally considered to be the most specific indicator of ER stress (personal communications, Drs David Ron; Kazutoshi Mori). We therefore examined the expression of BIP mRNA by Northern analysis in the chronic MPTP model at two time points: post-injection days 0 and 2. At neither time was BiP expression increased. In the adult 6OHDA model, we examined BiP expression by non-radioactive in situ hybridization. No induction was observed at 48 hours postlesion. Therefore there is no induction of this ER stress marker.

We also determined whether the XBP-1 422 bp splice variant could be identified in SN tissues in the acute or chronic MPTP or adult 6OHDA models. For this assessment, we used RNA derived from the kidney of a mouse treated with tunicamycin as a positive control. In the presence of this positive control, the XBP-1 splice variant was not detected in the chronic or acute MPTP models, or the 6OHDA model. There are two possible conclusions. It remains possible that ER stress is occurring in these models, but these markers thereof remain below the limit of detection in these studies conducted at the tissue (as opposed to cellular) level. The second possible conclusion is that CHOP is induced in these models not on the basis of ER stress, but rather some other cellular stress, such as oxidative stress.

All of this additional data related to the expression of BiP mRNA and the XBP-1 422 bp splice variant is included in the published manuscript.

Task 3. **To determine if caspase-12 is a mediator of 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

We completed experiments to study the effect of a homozygous null mutation for caspase-12 on sensitivity to death in the adult 6OHDA model. We have found that the null mutation does not reduce levels of apoptosis induced by 6OHDA: Wildtype mice: 4.1 ± 1.3 (apoptotic profiles/SN); Caspase-12 Null: 2.8 ± 1.0 (NS). In addition, we have found that the null mutation does not result in an increased survival of SN dopamine neurons. When examined at 4 weeks post 6OHDA lesion, the numbers of dopamine neurons determined by stereology are not different between the wildtype and null mice: Wild type: 4538 ± 110 (Tyrosine Hydroxylase-positive neurons/SN) (Control, Non-Lesioned side) and 1394 ± 263 (Experimental, 6OHDA-lesioned side) (a reduction of 69%); Null: 4533 ± 92 (Control) and 1350 ± 239 (Experimental, 6OHDA-lesioned side) (a reduction of 70%). Since there is substantial evidence that caspase-12 is a critical downstream mediator of PCD due to ER stress in rodent, these results would suggest that the expression of CHOP in the 6OHDA model is not mediated by ER stress, but rather oxidative stress. This conclusion is compatible with the results summarized above that other markers of ER stress were not identified in the 6OHDA model.

New Tasks:

(1) **To determine the molecular order of CHOP and JNK activation in 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

In separate work, funded by our Udall Parkinson's Disease Research Center of Excellence at Columbia University, we have found that the two c-jun N-terminal kinases, JNK2 and JNK3, are essential for PCD of dopamine neurons in the 6OHDA model. As shown in Figure 1 below, the combined JNK 2/3 double null mutation results in a complete abrogation of apoptosis, and a complete protection of these neurons. In relation to the work performed in this proposal, this result was unexpected because up to this time, there had been no postulated upstream control of CHOP by the JNK/c-jun kinase cascade, and so we anticipated that the cell death mediated by CHOP would persist in the JNK 2/3 null mice. Since this did not occur, we were led to ask, is JNK/c-jun activation upstream to CHOP activation? In this new Task, we addressed this question by determining the effect of the double JNK 2/3 null mutation on CHOP expression in the 6OHDA model. As shown in Figure 2, the combined JNK2/3 null mutations result in a decreased number of CHOP-positive profiles. We therefore conclude that that JNK2 and JNK3 are the principal, essential mediators of apoptosis in the 6OHDA model, and that they are operative upstream to CHOP. These Figures are based on a manuscript now in preparation.

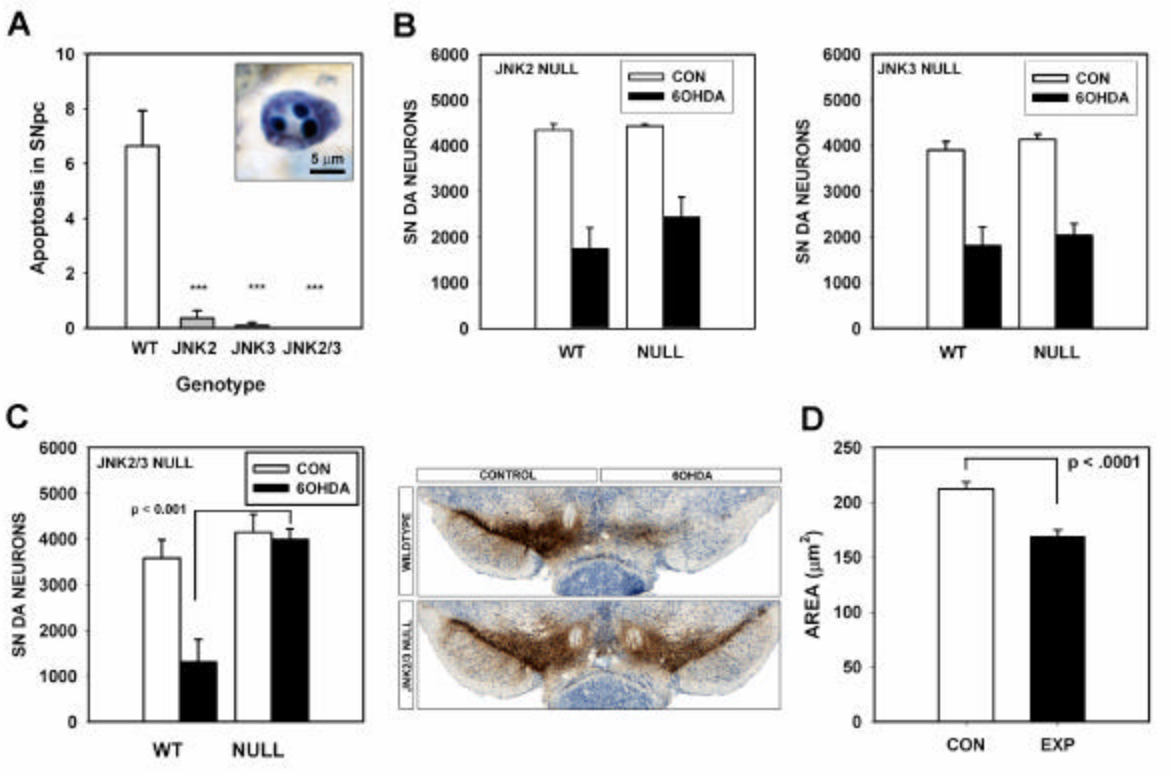

Fig. 1. Inhibition of apoptosis in homozygous *jnk2/jnk3* double null mice. (*A*) Adult mice of different genotypes were lesioned by intrastriatal injection of 6OHDA and sacrificed after 6 days for the counting of apoptotic profiles in the SNpc. A typical example of an apoptotic profile, with characteristic chromatin clumps, is demonstrated by thionin counterstain in the inset. The homozygous JNK2 and JNK3 single null mutations suppressed apoptosis by 95% and 98%, respectively, and the homozygous JNK2/3 double null mutations completely abrogated apoptosis (***$p < 0.001$, ANOVA, Wildtype (WT) in comparison to each of the JNK null genotypes; N = 7 each group, with the exception of JNK3 single null, N = 5). (*B*) The homozygous JNK2 and JNK3 single null mutations did not result in an increased survival of SN dopamine neurons, based on stereologic counts of TH-immunostained neurons at 28 days postlesion. The decreased number of TH-positive neurons in the SN ipsilateral to the 6OHDA injection in comparison to the contralateral, non-injected Control (CON) was not different between Wildtype (WT) mice and either JNK2 or JNK3 single null animals (N = 6, both genotypes in the JNK2 study; N = 6 Wildtype, N = 9 JNK3 null in the JNK3 study). (*C*) The homozygous JNK2/3 double null mutations provided almost complete protection of SN dopamine neurons, based on stereologic counts at 28 days postlesion (N = 7, both genotypes). Among Wildtype mice, there was a 63% loss of dopamine neurons, typical for this model, whereas among JNK2/3 nulls, there was only a 4% decrease, not a significant difference in comparison to the contralateral, non-injected Control (CON) side. The adjacent panel shows low power photomicrographs of representative TH-immunostained and thionin-counterstained anterior SN sections from Wildtype (WT) (top) and JNK2/3 nulls (bottom) at 28 days following unilateral 6OHDA injection. (*D*) Although there is an almost complete preservation of SN TH-positive neurons in the JNK2/3 null mice, they demonstrate a 20% loss of mean neuronal cross-sectional area ($p < 0.0001$, N = 100 neurons contralateral Control (CON) and ipsilateral 6OHDA Experimental (EXP) sides).

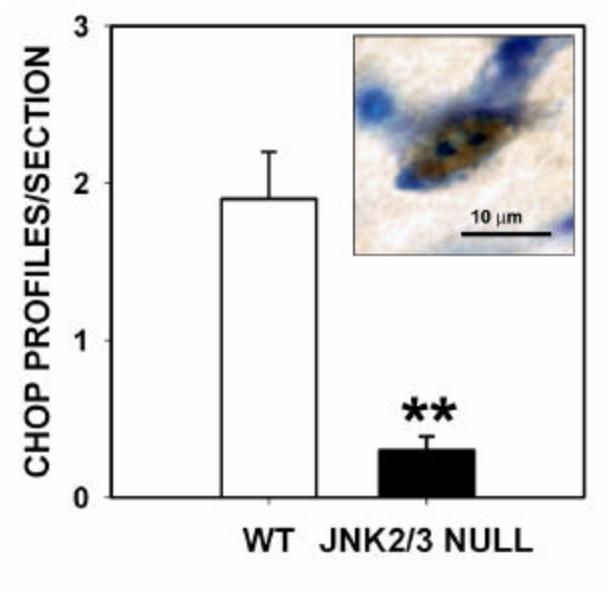

Fig. 2. CHOP-positive profiles are reduced in homozygous JNK2/3 double null mice following intra-striatal 6OHDA injection. At 6 days following intra-striatal injection of 6OHDA, JNK2/3 null mice were sacrificed and SN sections processed for CHOP immunostaining. An example of a thionin-counterstained neuronal profile, with intranuclear CHOP demonstrated by brown immunoreaction product, is shown in the inset. The number of such profiles was reduced by 85% in the JNK2/3 nulls in comparison to Wildtype (WT) controls (N = 4, each genotype; p = 0.003, t-test).

***(2)* To determine whether a novel AAV vector expressing the survival signaling kinase Akt can abrogate 6OHDA-induced apoptosis in DA neurons of the substantia nigra (SN) *in vivo*.**

Since this new Task demonstrated that the JNKs in the MAPK-signaling pathway are critical to abrogating apoptosis in dopamine neurons, and since the goal of this work ultimately is to attempt to develop new neuroprotective therapies for patients with Parkinson's disease, we explored the possibility of blocking PCD in dopamine neurons with an AAV vector expressing a constitutively active form of the survival signaling kinase Akt, which blocks MAPK pro-apoptotic signaling pathways by multiple mechanisms (reviewed in (3)). This work showed that a constitutively active form of Akt, Myr-Akt, can indeed effectively block PCD in SN dopamine neurons. A manuscript demonstrating this result is appended.

KEY RESEARCH ACCOMPLISHMENTS

-We have demonstrated that the transcription factor CHOP, a mediator of ER stress-induced apoptosis, is expressed in the most important neurotoxin models of parkinsonism: 6OHDA-induced apoptosis in postnatal and mature rats and mice, and MPTP-induced cell death following acute or chronic administration.

-We have demonstrated that CHOP plays an essential functional role in the adult model of 6OHDA-induced apoptosis. However, we have also found that CHOP does not play a role as a mediator of neuron death in a postnatal model of 6OHDA-induced death, because in that model death is mediated primarily by an axotomy effect.

-We have demonstrated that although CHOP is expressed in both the acute and chronic MPTP models, it does not play a role as a critical mediator of neuron death, because mice null for CHOP are not protected. Thus, we have demonstrated that there is an important fundamental difference between the adult 6OHDA and MPTP models of Parkinson disease: CHOP mediates death in the former, but not in the latter. We do not know at this time which, if either, of these models is closely related to death processes in human PD. However, this demonstrated difference in these models will provide a useful basis on which to evaluate them, as we learn more about the biochemical correlates of cell death in the human disease.

-In spite of the expression of CHOP in these models, there is no further evidence at the tissue level for the expression of other markers of ER stress, including BiP and the 422 bp splice variant of the transcription factor XBP-1. It is therefore possible that CHOP expression is due to oxidative stress rather than ER stress in these models. Alternatively, it is possible that these studies, conducted at the tissue level, lacked the sensitivity to detect changes at the cellular level. Future investigations will depend on the creation of reagents which will make possible detection of these markers at the cellular level.

-The caspase-12 homozygous null mutation does not protect from 6OHDA-induced neuron death. Since caspase-12 is an important mediator of PCD induced by ER stress, we conclude that the expression of CHOP in the 6OHDA model may not be due to ER stress, as also suggested by our inability to identify other ER stress markers, but rather to an oxidative stress.

-Although we have demonstrated that CHOP is an essential mediator of PCD in the adult 6OHDA model, double null mutations of the c-jun kinases JNK2 and JNK3 completely abrogate all apoptosis in this model. This result suggests that there is a heretofore unrecognized upstream control of CHOP by these kinases. This possibility has been supported by results in the current funding period.

-In the current funding period we have also demonstrated that the pro-survival kinase Akt has strong anti-apoptotic capabilities which can be demonstrated by viral vector transfer *in vivo*.

REPORTABLE OUTCOMES FUNDED BY THIS AWARD

A. Full Length Publications

(1) Original, Peer-reviewed Publications

Silva RM, Ries V, Oo TF, Yarygina O, Jackson-Lewis V, Ryu EJ, Lu PD, Stefan M. Marciniak, Ron D, Przedborski S, Kholodilov N, Greene LA, Burke RE. CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an *in vivo* neurotoxin model of parkinsonism. J Neurochemistry, 2005, 95:974-986.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During MJ, Kholodilov N, Burke RE. Oncoprotein Akt/PKB: Trophic effects in murine models of Parkinson's Disease. Proceedings of the National Academy of Sciences USA, 2006, 103:18757-18762.

Chen X, Rzhetskaya M, Kareva T, Bland R, During MJ, Tank AW, Kholodilov N, Burke RE. Anti-apoptotic and trophic effects of dominant negative forms of dual leucine zipper kinase (DLK) in dopamine neurons of the substantia nigra *in vivo.* Submitted, 2007.

Ries V, Silva RM, Oo TF, Cheng H-C, Kholodilov N, Flavell RA, Kuan C, Rakic P, Burke RE. JNK2 and JNK3 are essential for apoptosis of dopamine neurons of the substantia nigra, but are dispensable for their axon degeneration in a neurotoxin model of Parkinson's Disease, in preparation.

(2) Invited Reviews

Burke RE. Ontogenic cell death in the nigrostriatal system. In: Unsicker K (Ed). The Dopaminergic Nigrostriatal System: Development, Physiology, Disease. Cell and Tissue Research. 2004, 318:63-72. (The work done within this project on the role of CHOP in natural cell death in SN dopamine neurons was cited in the following recent review. Support by this award is acknowledged).

Silva RM, Kuan C-Y, Rakic P, Burke RE. The Mixed Lineage Kinase-c-Jun N-Terminal Kinase Signaling Pathway: A New Therapeutic Target in Parkinson's Disease. *Movement Disorders*, 2005, 20:653-664.

Kuan C-Y, Burke RE. Targeting the JNK signaling pathway for stroke and Parkinson's disease therapy. *Current Drug Targets CNS Neurol Disord*, 2005, 4: 63-7.

Burke RE. Programmed Cell Death In Parkinson's Disease. In: W. C. Koller and E. Melamed, The Handbook of Clinical Neurology: Parkinson's Disease and Related Disorders. Elsevier Limited, Edinburgh, UK, 2007.

Burke RE. GDNF as a candidate striatal target-derived neurotrophic factor for the development of substantia nigra dopamine neurons. In: P. Riederer, H. Reichmann, M.B.H. Youdim, and M. Gerlach (Eds), Parkinson's Disease and Related Disorders, SpringerWien, New York, 2006.

Burke RE. Programmed Cell Death. In S.A. Factor and W.J.Weiner (Eds), Parkinson's Disease: Diagnosis and Clinical Management, Demos Medical Publishing, Inc., New York, 2007, in press.

Oo TF and Burke RE. Histochemical Methods For The Detection Of Apoptosis In The Nervous System. In C.R. Gerfen (Ed), Current Protocols in Neuroscience, John Wiley and Sons, Inc, 2007.

Burke RE. Kinase Signaling Pathways: Potential Therapeutic Targets in Parkinson's Disease. *Future Neurology,* 2007, 2: 39-49.

Burke RE. Inhibition Of MAPK And Stimulation Of Akt Kinase Signaling Pathways: Two Approaches with Therapeutic Potential In The Treatment Of Neurodegenerative Disease. *Pharmacology and Therapeutics*, 2007, 114:261-277.

Ries V, Burke RE. Rodent Toxin Models of Parkinson's Disease: An Overview. In R Nass and S Przedborski, (Eds.), Parkinson's Disease: Pathogenic and Therapeutic Insights from Toxin and Genetic Models, Elsevier, San Diego, in press.

B. Abstracts

Silva RM, Oo TF, Jackson-Lewis VJ, Ryu E, Ron D, Przedborski S, Greene LA, Burke RE. The dopaminergic neurotoxins 6-hydroxydopamine (6-OHDA) and MPTP induce expression of CHOP (GADD153) in substantia nigra (SN) in vivo. Abstract, Society for Neuroscience, 2003.

Silva R, Ries V, Oo TF, Kholodilov N, Yarygina O, Jackson-Lewis V, Ryu E, Ron D, Przedborski S, Greene L, Burke RE. CHOP/Gadd153 Is A Mediator Of Apoptotic Death In Dopamine (DA) Neurons Of The Substantia Nigra (SN) In A Neurotoxin Model Of Parkinsonism. Sixth Annual Meeting of the NINDS Udall Centers for Parkinson Research, 2004.

Ries V, Kareva T, Rzhetskaya M, Bland R, During M, Kholodilov N, Burke RE. A constitutively active form of Akt protects dopamine neurons of the mouse substantia nigra in a neurotoxin model of Parkinson's disease. Society for Neuroscience, 2005.

Ries V, Kareva T, Rzhetskaya M, Bland R, During M, Kholodilov N, Burke RE. A constitutively active form of the oncoprotein Akt protects dopamine neurons of the substantia nigra in a neurotoxin model of Parkinson's disease. Seventh Annual Meeting of the NINDS Udall Centers for Parkinson Research, 2005.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During M, Kholodilov N, Burke RE. A constitutively active form of the oncoprotein AKT protects dopamine neurons of the substantia nigra in a neurotoxin model of Parkinson's disease. The World Parkinson Congress, 2006.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During M, Kholodilov N, Burke RE. Protection and repair of the nigrostriatal dopaminergic system by a constitutively active form of the oncoprotein AKT in a neurotoxin model of Parkinson's disease. The American Academy of Neurology, 2006.

Kholodilov NG, Rzhetskaya M, Burke RE. Analysis of the relative abundance of the mixed lineage kinases (MLK) in human substantia nigra (SN) using real-time PCR. Society for Neuroscience, 2006.

Ries V, Henchcliffe C, Kareva T, Rzhetskaya M, Bland R, During M, Kholodilov N, Burke RE. A constitutively active form of AKT mediates trophic effects on adult dopamine neurons of the mouse substantia nigra. Society for Neuroscience, 2006.

Chen X, Kareva T, Rzhetskaya M, Bland R, During MJ, Kholodilov N, Burke RE. Inhibition of dual-leucine-zipper-bearing kinase by AAV-mediated gene delivery of dominant negatives protects dopaminergic neurons in adult mice following 6-OHDA lesion. Society for Neuroscience, 2006.

CONCLUSIONS

We conclude that CHOP, a mediator of apoptosis due to ER stress, is upregulated in virtually all of the major neurotoxin models of parkinsonism. Our evidence indicates that CHOP is a functional mediator of apoptosis in the adult 6OHDA-induced model of parkinsonism. Our results in the MPTP model indicate that CHOP is not a mediator of cell death in this model, in spite of the fact that it is robustly upregulated.

We have found that two important markers of ER stress, the BiP chaperone protein, and the 422 bp splice variant of the transcription factor XBP-1, are not upregulated in the adult 6OHDA model, or the chronic or acute MPTP models. It is therefore likely that the upregulation of CHOP in these neurotoxin models is not mediated by ER stress, but rather another form of cellular stress, such as oxidative injury, which has been postulated to occur in both the 6OHDA and the MPTP models.

This latter conclusion is supported by data showing that homozygous null mutations in caspase-12, an important mediator of ER stress-induced apoptosis, do not protect in the 6OHDA model.

We have found that JNK2 and JNK3 are essential mediators of apoptosis in the 6OHDA model of PD.

We have found that viral vector-based neuroprotection of SN dopamine neurons can be achieved with the survival signaling kinase Akt.

REFERENCES

1. Ryu EJ, Harding HP, Angelastro JM, Vitolo OV, Ron D, Greene LA. Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. J Neurosci 2002;**22:**10690-10698.

2. Holtz WA, O'Malley KL. Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. J Biol Chem 2003;**278:**19367-19377.

3. Burke RE. Inhibition of MAPK and stimulation of Akt kinase signaling pathways: Two approaches with therapeutic potential in the treatment of neurodegenerative disease. Pharmacol Ther 2007;**114:**261-277.

APPENDIX

Robert E. Burke, MD

FINAL REPORT: DAMD17-03-1-0492

July 1, 2003 to June 30, 2007

Journal of Neurochemistry, 2005, **95**, 974–986 doi:10.1111/j.1471-4159.2005.03428.x

CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an *in vivo* neurotoxin model of parkinsonism

Robert M. Silva,* Vincent Ries,* Tinmarla Frances Oo,* Olga Yarygina,* Vernice Jackson-Lewis,* Elizabeth J. Ryu,§ Phoebe D. Lu,¶ Stefan J. Marciniak,¶ David Ron,¶ Serge Przedborski*,†,‡ Nikolai Kholodilov,* Lloyd A. Greene†,‡ and Robert E. Burke*,†

*Departments of *Neurology and †Pathology, ‡The Center for Neurobiology and Behavior, and §The Institute of Human Nutrition, The College of Physicians and Surgeons, Columbia University, New York, USA*

¶Departments of Medicine and Cell Biology, The Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, USA

Abstract

There is increasing evidence that neuron death in neurodegenerative diseases, such as Parkinson's disease, is due to the activation of programmed cell death. However, the upstream mediators of cell death remain largely unknown. One approach to the identification of upstream mediators is to perform gene expression analysis in disease models. Such analyses, performed in tissue culture models induced by neurotoxins, have identified up-regulation of CHOP/GADD153, a transcription factor implicated in apoptosis due to endoplasmic reticulum stress or oxidative injury. To evaluate the disease-related significance of these findings, we have examined the expression of CHOP/GADD153 in neurotoxin models of parkinsonism in living animals. Nuclear expression of CHOP protein is observed in developmental and adult models of dopamine neuron death induced by intrastriatal injection of 6-hydroxydopamine (6OHDA) and in models induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). CHOP is a mediator of neuron death in the adult 60HDA model because a null mutation results in a reduction in apoptosis. In the chronic MPTP model, however, while CHOP is robustly expressed, the null mutation does not protect from the loss of neurons. We conclude that the role of CHOP depends on the nature of the toxic stimulus. For 6OHDA, an oxidative metabolite of dopamine, it is a mediator of apoptotic death.

Keywords: apoptosis, endoplasmic reticulum stress, oxidative stress, Parkinson's disease, programmed cell death, substantia nigra.

J. Neurochem. (2005) **95,** 974–986.

There is an emerging consensus that programmed cell death (PCD) is likely to play a role in neuron death in neurodegenerative disease (Mattson 2000; Yuan and Yankner 2000). For Parkinson's disease (PD), this consensus is based on studies in animal models and human post-mortem material demonstrating either apoptotic morphology or immunohistochemical evidence for activation of caspases (reviewed in Vila and Przedborski 2003). One of the hallmarks of PCD is that in many contexts, it requires the transcription of genes that mediate cell death (Martin *et al*. 1988; Oppenheim *et al*. 1990). Therefore, a useful strategy to attempt to identify genes that mediate neuronal degeneration is to screen gene expression in models of disease. Such a strategy has been implemented for PD by performing serial analysis of gene

Received May 9, 2005; revised manuscript received July 6, 2005; accepted July 7, 2005.

Address correspondence and reprint requests to Robert E. Burke, Department of Neurology, Room 308, Black Building, Columbia University, 650 West 168th Street, New York, NY 10032, USA.
E-mail: rb43@columbia.edu

Abbreviations used: ABC, avidin-biotinylated-horseradish peroxidase complexes; ER, endoplasmic reticulum; MFB, medial forebrain bundle; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; NRISH, non-radioactive *in situ* hybridization; 6OHDA, 6-hydroxydopamine; PB, phosphate buffer; PBS, phosphate-buffered saline; PCD, programmed cell death; PD, Parkinson's disease; PLD, post-lesion day; PND, post-natal day; SSC, saline sodium citrate; SN, substantia nigra; TBS, Tris-buffered saline; TH, tyrosine hydroxylase.

expression in PC12 cells, a catecholaminergic cell line (Greene and Tischler 1976), treated with 6-hydroxydopamine (6OHDA), a neurotoxin which is an oxidative metabolite of endogenous dopamine (Senoh and Witkop 1959; Kostrzewa and Jacobowitz 1974). Among the up-regulated transcripts identified by this analysis, and of particular potential relevance to neuronal death, was a striking induction of the transcription factor CHOP/GADD153 (Ryu *et al*. 2002). CHOP has been implicated as a mediator of apoptosis in the contexts of both endoplasmic reticulum (ER) stress (Matsumoto *et al*. 1996; Zinszner *et al*. 1998; Kawahara *et al*. 2001; Maytin *et al*. 2001; Gotoh *et al*. 2002; Oyadomari and Mori 2004) and oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002). In keeping with a possible role of either of these forms of cellular stress in mediating CHOP induction and neuron death, the analysis of gene expression also identified the induction of many other genes involved in ER and oxidative stress (Ryu *et al*. 2002, 2005).

A similar induction of CHOP was also observed by Holtz and O'Malley in a gene expression screen of neurotoxin models of parkinsonism (Holtz and O'Malley 2003). These investigators used Affymetrix gene arrays to screen dopaminergic MN9D cells following exposure to either 6OHDA or MPP^+, and noted that the most highly expressed transcript, for both neurotoxins, was that for CHOP (Holtz and O'Malley 2003).

These findings in gene expression screens performed *in vitro* are potentially relevant to human PD because the classes of transcripts induced, those related to oxidative stress and ER stress, relate to important current hypotheses for pathogenesis. The possibility that the oxidative metabolism of dopamine may be injurious to dopaminergic neurons is one of the longest-standing hypotheses (Fahn and Cohen 1992). More recently, ER stress has been postulated to play a role. An important genetic cause of PD is loss of function mutations in *parkin* (Ishikawa and Tsuji 1996; Kitada *et al*. 1998). These mutations have been implicated in abnormal protein processing because parkin is an E3 ubiquitin-ligase (Shimura *et al*. 2000) and, as such, it plays a role in targeting cellular proteins for destruction by the proteasome (Ciechanover 1998). One putative protein target of parkin, Pael-R, is a difficult-to-fold protein, and it has been postulated that its accumulation may result in dopaminergic neuron death due to ER stress (Imai *et al*. 2000, 2001).

The possible implications of these *in vitro* observations for the pathogenesis of PD depend on whether they generalize to the *in vivo* context. We have therefore investigated the expression of CHOP in several neurotoxin models of parkinsonism in living animals: substantia nigra (SN) dopamine neuron degeneration induced by intrastriatal injection of 6OHDA in both developing (Marti *et al*. 1997) and adult rodents (Sauer and Oertel 1994), and by both the acute (Heikkila *et al*. 1984) and chronic (Tatton and Kish 1997) systemic administration of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). In addition, we have sought to determine whether CHOP plays a functional role as an essential mediator of dopamine neuron death by examining the vulnerability of homozygous CHOP null mice.

Materials and methods

Animals

For the study of postnatal rats, timed pregnant females were obtained from Charles River Laboratories (Wilmington, MA, USA). The date of delivery was defined as postnatal day (PND) 1. For adult mouse studies utilizing the 6OHDA and MPTP models, C57BL/6 mice were obtained from Charles River. CHOP null mice were produced by homologous recombination to replace all of the CHOP coding sequence (except for the final 34 C-terminal residues) with the coding sequence for β-galactosidase containing a nuclear localization signal. The neomycin selection cassette was then removed by Cre recombinase. There was no detectable CHOP protein in cells and tissues derived from these animals (Fig. 1). These mice were back-crossed into the C57BL/6 strain five times before breeding for experiments. The CHOP null mice were genotyped by PCR analysis of tail DNA using three-primer PCR analysis as previously described (Zinszner *et al*. 1998), with the modification that the primer to detect the mutant allele was based on the β-galactosidase sequence and produced a 300 bp product.

Animal models

The models used in this investigation are summarized in Table 1. The 6OHDA model in postnatal rats was performed as previously described (Marti *et al*. 1997). Briefly, rat pups at PND7 were pretreated with 25 mg/kg desmethylimipramine, anesthetized by hypothermia and placed prone on an ice pack. 6-OHDA hydro-

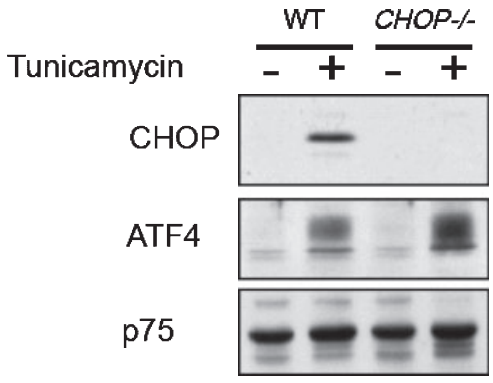

Fig. 1 Absence of CHOP protein expression in CHOP null mice. Immunoblot of nuclear extract of untreated and tunicamycin-treated (2 μg/mL, 6 h) wild-type and CHOP–/– cells blotted with antisera reactive with CHOP, ATF4 (a positive control) and p75, a ubiquitously-expressed nuclear protein that serves as a loading marker. No protein CHOP expression is observed in CHOP null cells after tunicamycin treatment. The antibody to ATF4 was raised against a full-length bacterially-expressed fusion protein and is characterized in Ron and Habener (1992). The p75 band was detected by an antiserum to *Drosophila* protein, described in Immanuel *et al.* (1995).

Table 1 Models used to assess the role of CHOP/GADD153 in apoptosis in SN dopamine neurons

Treatment	Species	Age	Route	Morphology of cell death
None (natural cell death)	Rat	Developmental	N.A.	Apoptosis
Axotomy	Rat	Developmental	N.A.	Apoptosis
6OHDA	Rat or Mouse	Developmental	Intrastriatal	Apoptosis
6OHDA	Mouse	Adult	Intrastriatal	Apoptotic and non-apoptotic
MPTP	Mouse	Adult	I.P., acute	Non-apoptotic
MPTP	Mouse	Adult	I.P., chronic	Apoptotic and non-apoptotic

Abbreviations: N.A., not applicable; I.P., intraperitoneal.

bromide (Regis, Morton Grove, IL, USA) was prepared at 15 μg (total weight)/1.0 μL in 0.9% NaCl/0.02% ascorbic acid, and infused by pump (Harvard Apparatus, Holliston, MA, USA) at a rate of 0.25 μL/min for 4 min (total dose 15 μg). Postnatal mice were injected in a similar fashion except that the solution was prepared at a concentration of 20 μg/μL and infused for 2 min, for a total dose of 10 μg. For experiments in postnatal mice, littermate wild-type and heterozygote animals were examined in comparison with nulls. Adult mice were infused with a concentration of 5 μg/μL at a rate of 0.5 μL/min for 8 min for a total dose of 20 μg. For experiments in adult mice, C57BL/6 adults were used as controls.

The medial forebrain bundle (MFB) axotomy model in postnatal rats was performed as previously described (El-Khodor and Burke 2002). Briefly, rat pups were anesthetized by hypothermia. Animals were positioned in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA, USA) to conform with the neonatal brain atlas of Heller *et al*. (1979). The MFB was transected by lowering a retractable wire knife (Kopf Instruments) through a skull burr hole 1.4 mm posterior and 2.5 mm lateral to bregma to a ventral position of 6.5 mm below bregma.

For the acute MPTP lesion model, mice received four i.p. injections of MPTP-HCl (20 mg/kg free base; Sigma, St Louis, MO, USA) dissolved in saline, 2 h apart in 1 day as previously described (Teismann *et al*. 2003). Control mice received saline only. MPTP handling and safety measures were in accordance with our published guidelines (Przedborski *et al*. 2001). For the chronic MPTP model, mice received one i.p. injection of MPTP-HCl per day (30 mg/kg per day of free base) for 5 consecutive days as described (Tatton and Kish 1997).

All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University.

Immunohistochemistry

For CHOP immunoperoxidase histochemistry, animals were perfused intracardially first with 0.9% NaCl and then with 4% paraformaldehyde and 0.1 M phosphate buffer (PB). The brains were then removed and post-fixed in the same fixative for 3 h. Each brain was then cryoprotected in 20% sucrose for 24 h. The brains were then rapidly frozen in isopentane on dry ice, and sections were cut in a cryostat at 30 μm. Sections were processed free-floating. After a phosphate-buffered saline (PBS) wash and treatment with PBS, 0.5% bovine serum albumen and 0.1% Triton X-100, sections were incubated with rabbit anti-CHOP at 1 : 500 for 48 h at 4°C. After a wash, sections were then incubated with biotinylated protein A (prepared in this laboratory) at 1 : 100 for 1 h at ambient room temperature. Sections were further incubated with avidin-biotinylated-horseradish peroxidase complexes (ABC; Vector Laboratories, Burlingame, CA, USA) at 1 : 600 for 1 h. After incubation with diaminobenzidine, sections were mounted onto subbed slides and counterstained with thionin. The primary antibody had been previously characterized and used for immunohistochemistry (Ron and Habener 1992; Zinszner *et al*. 1998). For immunofluorescence double-labeling for CHOP and tyrosine hydroxylase (TH), sections were collected into Tris-buffered saline (TBS) and then treated with TBS/0.2% Triton/2% goat serum/2% horse serum. They were then incubated in the same solution with anti-CHOP (1 : 500) and mouse anti-TH (1 : 40) (Chemicon, Temecula, CA, USA) for 48 h at 4°C. The sections were next treated with Texas red horse anti-mouse (Vector) at 1 : 75 and biotinylated goat anti-rabbit (Vector) at 1 : 75 for 1 h at ambient room temperature, followed by treatment with Fluor-avidin (Vector) at 1 : 100 for 1 h. Sections were then mounted onto gelatin-coated glass slides and coverslipped with Dako antifade medium (Carpinteria, CA, USA). The sections were examined by epifluorescence with a Nikon Eclipse 800 microscope.

For TH immunoperoxidase histochemistry, animals were perfused, as described above, and then post-fixed in the same fixative for 1 week. Each brain was cryoprotected in 20% sucrose for 24–48 h and then rapidly frozen. A complete set of serial sections through the SN was cut at 30 μm. Sections were saved individually in serial order at 4°C, and individual sections at regular intervals were then selected for TH immunostaining, in conformity with the fractionator method of sampling (Coggeshall and Lekan 1996) (see below). Sections were processed free-floating, as described above for CHOP. The primary antibody was a rabbit anti-TH (Calbiochem, La Jolla, CA, USA) at 1 : 1000. After treatment with biotinylated protein A and ABC, sections were mounted on subbed slides in serial order and thionin-counterstained.

Quantitative morphology

For the analysis of the time course of appearance of CHOP-positive nuclear profiles and apoptosis in the postnatal 6OHDA model in rats, counts were performed as previously described (Oo *et al*. 2003; Ganguly *et al*. 2004). CHOP-positive nuclear profiles were counted in identical fashion on the same sections.

The number of SN dopaminergic neurons in the lesion experiments with CHOP null and C57BL/6 control mice was

determined by stereological analysis. A complete set of TH-immunostained serial sections, sampled as every fourth section through the SN, was analyzed by a stereological method for each animal. Each analysis was performed under blinded conditions on coded slides. For each animal, the SN on each side of the brain was analyzed. For each section, the entire SN was identified as the region of interest. Using StereoInvestigator software (Micro Bright Field, Inc., Williston, VT, USA) a fractionator probe was established for each section. The number of TH-positive neurons in each counting frame was then determined by focusing down through the section, using a 100× objective under oil, as required by the optical dissector method (Coggeshall and Lekan 1996). Our criterion for counting an individual TH-positive neuron was the presence of its nucleus either within the counting frame, or touching the right or top frame lines (green) but not touching the left or bottom lines (red). The total number of TH-positive neurons for each SN on one side was then determined by the StereoInvestigator program. The total volume of the SN was also determined by the StereoInvestigator program for each brain on the basis of the sum of volumes derived from the area of each individual serial section and the tissue height represented by that section.

Northern analysis and non-radioactive *in situ* hybridization analysis (NRISH) of BiP

Rat BiP cDNA was subcloned into pCMS-EGFP (BD Biosciences, San Jose, CA, USA) as described (Ryu *et al*. 2002) and used for creation of an antisense RNA probe. Northern analysis was performed as previously described (El-Khodor *et al*. 2001). Briefly, RNA was isolated from microdissected SN using the Qiagen RNAeasy Mini kit (Valencia, CA, USA). The RNA concentration of each sample was determined by measuring absorption at 260 nm on a GenQuant spectro-photometer (Amersham Pharmacia Biotech, Piscataway, NJ, USA). A 20 μg aliquot of each RNA was electrophoresed in 1.4% agarose-formaldehyde gel and transferred onto an Immobilon (+) membrane (Millipore, Bedford, MA, USA). The hybridization was performed overnight at 68°C in Ultrahyb buffer from Ambion (Austin, TX, USA). The membrane was then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics, Indianapolis, IN, USA).

For NRISH, brains were rapidly removed from 6OHDA-injected adult mice at 48 h post-injection, and rapidly frozen in embedding medium on dry ice. Sections (14 μm) were thaw-mounted on glass slides (Superfrost Plus, Fisher, Hampton, NH, USA). For hybridization, sections were warmed on a slide warmer at 37°C for 20 min, and then fixed by immersion in 4% paraformaldehyde in 0.1 M PBS. After washing, sections were acetylated by treatment with acetic anhydride in triethanolamine. After another wash, sections were treated with a pre-hybridization solution, as previously described (Burke *et al*. 1994), for 2 h at ambient room temperature. Sections were then covered with hybridization solution and incubated overnight at 68°C in a humidified chamber. Hybridization solution contained the BiP riboprobe labeled with digoxigenin-UTP (1 μL/slide) (200–400 ng/mL), prepared as per the manufacturer's instructions (Roche Diagnostics, Penzberg, Germany). The size and integrity of labeled probe were confirmed by gel electrophoresis. The same probe used for northern analysis was used for the *in situ* hybridization. After a wash in 0.5× saline sodium citrate (SSC) for 10 min, followed by a wash in 0.2× SSC at 68°C for 30 min, sections were incubated with an anti-digoxigenin antibody (Roche) at 1 : 5000 overnight at 4°C. After additional washes, sections were incubated with a solution containing nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Promega Corporation, Madison, WI, USA) in a darkened humidified chamber overnight. Sections were then washed and coverslipped with Dako aqueous mounting medium.

RT-PCR/Southern blot analysis of the XBP-1 splice variant

To perform Southern analysis of the x-box binding protein-1 (XBP-1) splice variant, we first generated a DNA probe. We performed reverse transcription using RNA isolated from mouse kidney after treatment with tunicamycin. We then performed PCR of the 422 bp region of mouse XBP-1 containing the site of the unconventional splice, using primers based on nucleotide number 363 (Accession no. BC029197) (5′- CCTTGTGGTTGAGAACCAGG-3′) (forward) and nucleotide number 810 (5′-GAGGCTTGGTGTATACATGG-3′) (reverse). The band containing the spliced DNA fragment of XBP-1 was isolated from an agarose gel, subcloned in the pGEM-T vector (Promega) and sequenced. The DNA fragment containing the site of the XBP-1 unconventional splice was isolated from this clone using SalI and NcoI restriction enzymes (Promega). This fragment was then used to generate a ^{32}P-labeled DNA probe with the Rediprime II Kit, random prime labeling system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). For XBP-1 splice variant Southern blot analysis, RNA was isolated from tissues using the Qiagen RNAeasy Mini Kit, as described above. First strand cDNA was then synthesized from isolated RNA by the RT system (Promega). PCR was performed individually with each cDNA sample using the above primers with Taq polymerase from Roche. A 10 μg aliquot of each DNA sample was electrophoresed in a 2% agarose gel. The DNA was then transferred onto a Hybond-N membrane (Amersham Pharmacia Biotech), hybridized with the XBP-1 DNA probe in Ultrahyb solution (Ambion) overnight at 42°C, washed as recommended, then exposed to phosphorimager cassettes, scanned and analyzed by Image Quant software (Molecular Dynamics).

Statistical analysis

The time course of appearance of apoptotic and CHOP-positive profiles in the postnatal 6OHDA model was analyzed by ANOVA with a Tukey post hoc analysis. Stereological determination of the number of SN dopaminergic neurons in the 6OHDA and MPTP lesion experiments was analyzed by ANOVA with a Tukey post hoc analysis. The number of apoptotic profiles in wild-type and CHOP null adult mice in the 6OHDA model was analyzed by the *t*-test. All statistical analyses were performed using SigmaStat software (SPSS Science, Chicago, IL, USA).

Results

CHOP protein expression is induced in a developmental neurotoxin model of parkinsonism

We initially performed *in vivo* experiments in a rat developmental model in which the intrastriatal injection of 6OHDA results in the induction of death in dopamine neurons of the SN, exclusively with the morphology of

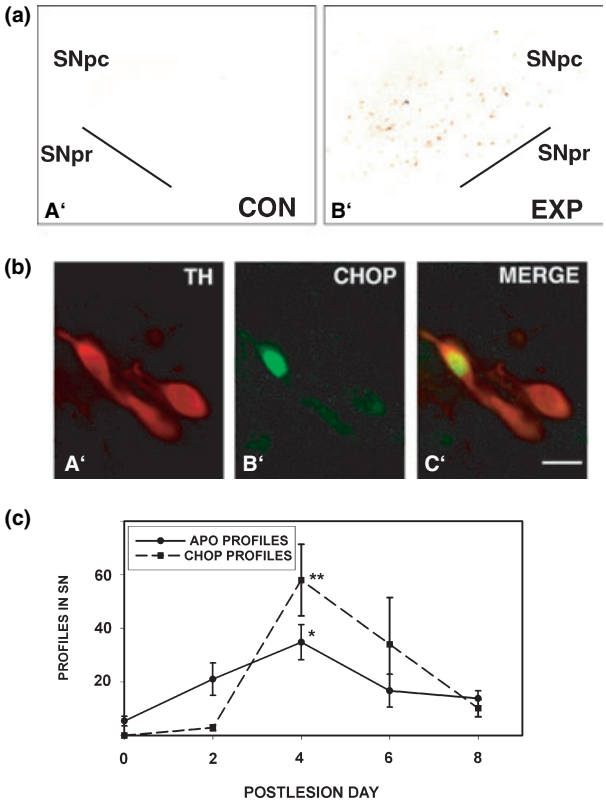

Fig. 2 Localization and time course of CHOP expression following developmental 6OHDA lesion in postnatal rats. (a) Low power photomicrographs at PLD6 of the substantia nigra contralateral (control: Con) and ipsilateral (experimental: Exp) to an intrastriatal injection of 6OHDA in a PND7 rat. CHOP protein expression is demonstrated by immunoperoxidase staining without a counterstain. CHOP-positive nuclei therefore appear as punctate brown profiles at this power. On the contralateral control side (a′), there is an absence of staining. On the ipsilateral experimental side, numerous CHOP-positive profiles are observed within the SNpc (b′). No positive profiles were observed in the SNpr or in the midbrain dorsal to the SNpc. (b) Double-immunofluorescence labeling for CHOP and TH in the SNpc at PLD4 following intrastriatal injection of 6OHDA in a PND7 rat. TH immunostaining is demonstrated by Texas Red (a′), CHOP by fluorescein (b′), and the merged image is shown in c′. CHOP immunostaining was predominantly nuclear. Following 6OHDA injection, CHOP staining was observed strictly within TH-positive, dopaminergic profiles of the SNpc. Note that CHOP-positive profiles appear normal morphologically; there is no apparent change in neuronal shape or proximal dendrites in comparison with adjacent, CHOP-negative, TH-positive neurons. Bar in c′ = 10 μm. (c) Time course for the appearance of apoptotic and CHOP-positive profiles in SN following intrastriatal injection of 6OHDA in PND7 rats. A total of 24 rats was studied: $n = 4$ at PLD0 and 2; $n = 5$ at PLD4 and 6; $n = 6$ at PLD8. CHOP-positive and apoptotic profiles were counted in the same sections from each animal, as described in Methods. The number of CHOP-positive profiles reached a peak at PLD4 (**$p < 0.02$ vs. PLD0, 2 and 8; ANOVA, Tukey post hoc). The number of apoptotic profiles also reached a peak at PLD4 (*$p < 0.05$ vs. PLD0 and 8; ANOVA, Tukey post hoc). However, the time of induction for the two types of profile differed at PLD2; for apoptotic profiles, the number at PLD2 was induced and not significantly different from the number at peak, whereas for CHOP profiles, there was no induction at PLD2. As discussed in the text, this difference may suggest that there are non-CHOP-dependent, as well as CHOP-dependent mechanisms of cell death in this model.

apoptosis (Marti *et al*. 1997). In this model, the unilateral intrastriatal injection of 6OHDA resulted in the unilateral induction of CHOP protein expression, demonstrated by immunohistochemistry (Fig. 2a). On the side of injection, CHOP expression was observed only in the SNpc, the exclusive site of neuron death in this model (Marti *et al*. 1997). CHOP expression was characterized at a cellular level by performing double-label immunofluorescence for CHOP and TH, to identify dopaminergic neurons of the SN. This analysis revealed that CHOP was expressed predominantly in the nucleus (Figs 2b,b′). To determine the cellular sites of CHOP expression within the SNpc, we examined 50 representative CHOP-positive nuclear profiles among six sections derived from two animals. This analysis showed that all CHOP-positive nuclei were within TH-positive, dopaminergic neurons of the SNpc. Thus, there was a precise correlation at the cellular level between the neuronal population that undergoes death in this model, and CHOP expression (Fig. 2b). All of the CHOP- and TH-positive neuronal profiles identified by the double-labeling procedure had a normal neuronal morphology: abundant cytoplasm, with a polygonal shape, and tapered proximal dendrites. We know from previous studies of this model that the vast majority of dopamine neurons die (Marti *et al*. 1997) and therefore, CHOP-positive profiles (all of which were TH-positive) are exceedingly likely to be destined to die. We therefore interpret the normal-appearing morphology to mean that if CHOP is to be implicated as a death mediator, it is expressed early in the death process, before any morphological change at the cellular level.

We investigated the time course of CHOP expression at the population level in this model. We recognize that since apoptosis occurs rapidly (Oppenheim 1991), and since at any given time of killing of the animal there will be a heterogeneous population of dying cells in varying stages of the death process, this population analysis will not resolve the cellular sequence of events. Nevertheless, it is informative to determine whether, at the population level, the appearance of CHOP-positive profiles correlates with the appearance of apoptotic profiles. CHOP expression at the population level in this model correlated at most times with the induction of apoptotic death (Fig. 2c). The occurrence of the peak number of CHOP-positive nuclear profiles corresponded precisely with the occurrence of the peak number of apoptotic profiles at postlesion day (PLD) 4. However, one exception to this correlation was that apoptosis was induced as early as PLD2, in the absence of any induction of CHOP, suggesting that an early component of apoptosis in this model is not associated with CHOP induction, as discussed further below.

Having demonstrated a co-localization between CHOP expression and the dopaminergic neuronal phenotype, and a temporal correlation between CHOP expression and apoptosis in the SN, we next examined the generality of the relationship in other developmental models in which apoptosis occurs. During the postnatal development of SN dopamine neurons, there is naturally-occurring cell death, exclusively with the morphology of apoptosis (Janec and Burke 1993; Oo and Burke 1997). Immunostaining for CHOP was performed on SN sections obtained from PND 14 rats (during the second phase of naturally-occurring cell death). We examined 36 SN sections among $n = 4$ rats and no instance of CHOP positivity was identified. Among these sections, 124 apoptotic profiles were identified, due to natural cell death (Fig. 3a). This naturally-occurring cell death can be augmented by an axotomy lesion of the medial forebrain bundle during the postnatal period (El-Khodor and Burke 2002). Examination of 18 SN sections from three PND6 rats at 24 h post-axotomy failed to reveal any CHOP-positive profiles (Fig. 3b). Among these sections, numerous apoptotic profiles were identified in SN, as described (El-Khodor and Burke 2002), and sections from 6OHDA-treated animals processed in parallel were positive for CHOP (Fig. 3c). Thus, we conclude that in the postnatal developmental period, CHOP protein expression is induced by the neurotoxin 6OHDA, but not by naturally-occurring cell death or a physical lesion that augments it.

CHOP protein expression in adult neurotoxin models of parkinsonism

To investigate the expression of CHOP in adult neurotoxin models, we exclusively studied mice to permit comparison between the 6OHDA model and the widely used MPTP mouse model of parkinsonism (Heikkila *et al.* 1984; Przedborski and Vila 2003). Adult mice injected into the striatum with 6OHDA demonstrated numerous CHOP-positive nuclei within neurons of the SNpc (Fig. 3d). For the study of MPTP effects on CHOP expression, we evaluated two dose regimens in common current use. Most widely used is an acute set of injections, 20 mg/kg for four doses, 2 h apart on a single day. This dosing regimen induces SN dopamine neuron death in the absence of apoptotic morphology (Jackson-Lewis *et al.* 1995). A second regimen utilizes a chronic set of injections, 30 mg/kg daily for 5 days (Tatton and Kish 1997), and results in neuron death with the morphological characteristics of apoptosis. In both of these

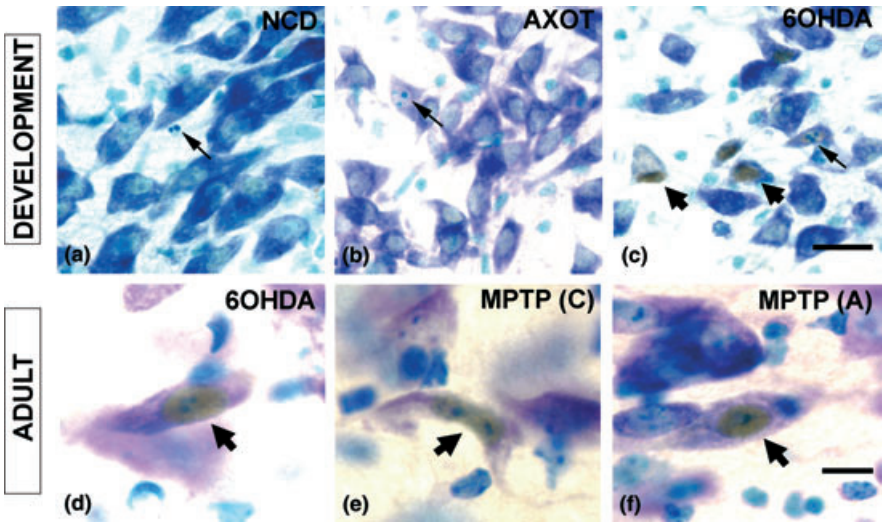

Fig. 3 CHOP is expressed in neurotoxin models of induced death in SN dopamine neurons. CHOP immunoperoxidase histochemistry was performed on free-floating sections, as described in Methods, with rabbit anti-CHOP (Zinszner *et al.* 1998) at 1 : 500 for 48 h, followed by thionin counterstain. (a) CHOP expression does not occur in SN during the apoptotic postnatal natural cell death event. A representative field showing a single apoptotic profile (arrow) in a PND14 rat is negative for CHOP immunostaining. (b) The naturally-occurring cell death event in SN can be augmented by postnatal axotomy of the dopaminergic axonal projection (El-Khodor and Burke 2002), as it is for many other developing neural projections (Oppenheim 1991). As for natural cell death, CHOP expression does not occur in this context, as shown by a representative field in a PND6 rat at 1 day post-lesion. An apoptotic profile is shown (arrow). (c) Unlike naturally-occurring cell death and axotomy, cell death induced by 6OHDA in PND7 rat results in the expression of CHOP in many neuronal profiles in the SNpc (broad arrowheads). In this model, CHOP-positive profiles rarely show basophilic apoptotic chromatin clumps (narrow arrow) (2% of instances). As discussed in the text, this rare association between CHOP expression and apoptotic nuclear morphology suggests that if CHOP is implicated in mediating death, it is likely to be an early participant, typically before morphological change. Bar = 20 μm for a, b and c. (d) A representative neuronal profile with a CHOP-positive nucleus (broad arrow) is shown at PLD6 following intrastriatal injection of 6OHDA in an adult mouse. (e, f) CHOP nuclear staining is also observed in SNpc neurons following MPTP injection in adult mice by either the chronic (C) or acute (A) regimens. Bar = 10 μm for d, e, f.

MPTP models, numerous CHOP-positive neuronal profiles were identified within the SN (Figs 3e and f). In all of these adult contexts, positive nuclear CHOP expression was identified in neurons which otherwise appeared normal, suggesting, as previously discussed, that if CHOP is to be implicated as a death mediator in these models, then it is expressed prior to degenerative morphological changes. We conclude from these studies that CHOP is generally expressed in the SNpc in neurotoxin models of parkinsonism.

CHOP mediates neuron death in the adult 6OHDA model

Having demonstrated close relationships between CHOP expression and the death of SN dopamine neurons in these neurotoxin models, we next sought to determine whether CHOP plays a critical functional role in mediating this death, as it has been shown to do in non-neuronal models of cell death due to ER stress (Zinszner *et al*. 1998) and oxidative stress. For this assessment, we compared the sensitivity of homozygous CHOP null mice with wild-type controls in their degree of sensitivity to neurotoxin-induced neuron death. In the postnatal 6OHDA model, we found that there was no difference between homozygous CHOP nulls and either heterozygous mice or wild-type controls in the degree of apoptosis among SN dopaminergic neurons induced by intrastriatal 6OHDA (Fig. 4). However, we recognized that in this model, death is known to be mediated not only by the direct effect of the neurotoxin but also, in the developmental period, by an 'axotomy' effect due to destruction of dopaminergic terminals during a period of target dependence (Marti *et al*. 1997). Since we had shown directly that axotomy does not induce CHOP expression, we considered the possibility that this admixture of death mechanisms may obscure a role played by CHOP in death due to the neurotoxin. Such a possibility was also suggested by the time course analysis in Fig. 2(c), which showed an early apoptotic component in the absence of CHOP induction. We therefore examined the sensitivity of adult CHOP null mice to intrastriatal injection of 6OHDA, as adult dopamine neurons do not have target dependence (Kelly and Burke 1996).

In adult mice, there was a clear protective effect of the homozygous CHOP null mutation (Fig. 5). CHOP null animals demonstrated a 65% reduction in the number of apoptotic profiles in the SNpc at the sixth post-lesion day. To determine whether this reduction in the magnitude of neuron death resulted in a lasting protection from the neurotoxin, we examined the number of surviving TH-positive neurons in the SN at 28 days post-lesion. This analysis revealed that the null mutation did provide a substantial, lasting protective effect; there was a 79% increase in the number of surviving TH-positive neurons in comparison with wild-type controls (control: 857 ± 131; null: 1531 ± 173 neurons per SN) (Fig. 5b). Nevertheless, the absolute magnitude of the protective effect in the nulls, expressed as 31% survival,

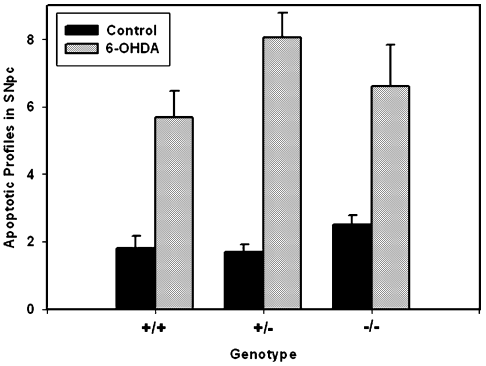

Fig. 4 The CHOP null mutation does not protect from induction of apoptosis in the developmental 6OHDA model. In total, 20 PND6 mice (wild-type $n = 5$; heterozygous $n = 10$; null $n = 5$) received a unilateral intrastriatal 6OHDA injection and were killed at PLD4 for the determination of apoptotic profiles within the TH-immunostained substantia nigra, as described in Methods. In all three genotypes, there was a robust induction of apoptosis, as previously described for rats (Marti *et al.* 1997) (ANOVA $p < 0.001$ for the 6OHDA effect). There were, however, no differences among the genotypes for this effect.

while significantly greater than that in the wild-type (19%, $p < 0.02$), was considerably less than anticipated based on a 65% suppression of apoptotic death in the acute period. In addition, at 28 days post-lesion, there was no evidence for sparing of dopaminergic innervation of the striatum in the nulls. In the nulls, there was a 28.0 ± 3.2 sparing of the optical density of TH-positive fibers within the striatum, as there was in wild-type controls (28.3 ± 3.6).

CHOP mediates a cellular response to injury, but not neuron death, in the chronic MPTP model

Given that CHOP expression is induced in both the acute and chronic MPTP models, we sought to determine whether it plays a role as a death mediator, as it does in the adult 6OHDA model. Since the role of CHOP as a death mediator has previously been identified in non-neuronal cells in the context of apoptosis (Zinszner *et al*. 1998), we examined its role in the chronic MPTP model in which apoptosis has been identified (Tatton and Kish 1997). Based on our results in the adult 6OHDA model demonstrating a disparity between the ability of the CHOP null mutation to protect from death in the acute period following the lesion as compared with the chronic period, we conducted separate assessments of both of these post-lesion periods. We found that the CHOP null mutation provided a protective effect in the acute (PLD4) period following the chronic administration of MPTP. The CHOP null animals demonstrated only a non-significant trend for a decrease in the number of TH-positive SN neurons at this time, whereas wild-type controls demonstrated a 65% decrease (Figs 6a and b). However, this difference could not be attributed to a difference in the magnitude of apoptotic death between the two genotypes. While there was

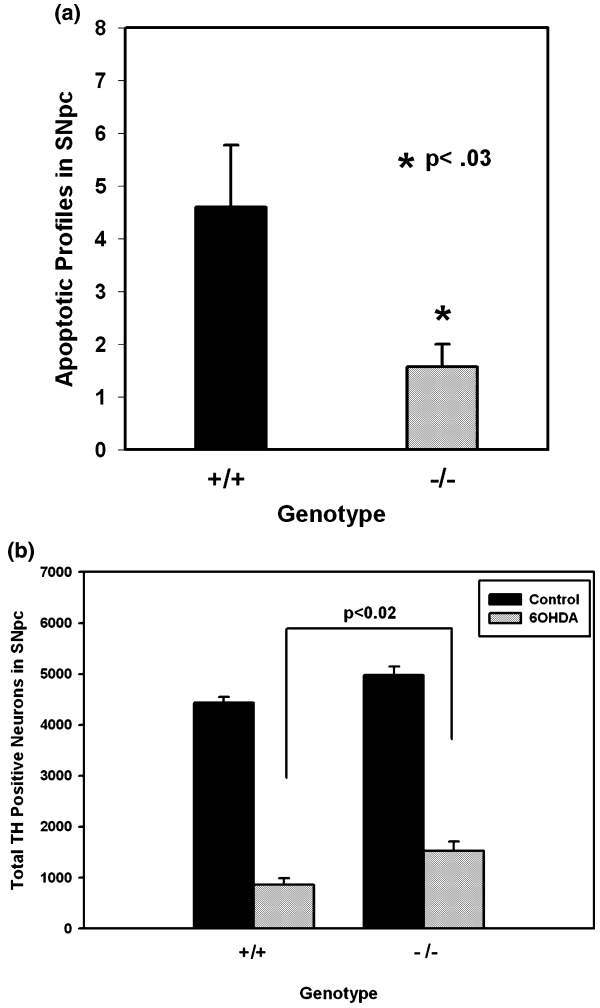

Fig. 5 The CHOP null mutation protects from apoptotic cell death in the adult 6OHDA model. (a) Wild-type ($n = 5$) and CHOP homozygous null ($n = 6$) adult mice were injected into the striatum with 6OHDA. They were killed 6 days later for TH immunostaining of the SN and counting of apoptotic profiles within the SNpc. The CHOP null animals demonstrated a 65% reduction in the level of apoptosis ($p < 0.03$, *t*-test). (b) Wild-type ($n = 7$) and CHOP null ($n = 8$) adult mice were injected with 6OHDA and killed 28 days later for TH immunostaining of serial sections for stereologic determination of the number of surviving dopaminergic neurons. In both genotypes, the 6OHDA injection led to a significant reduction in the number of SN dopamine neurons ($p < 0.001$, ANOVA; Tukey post-hoc). In the CHOP null animals, there was a 79% increase in the number of surviving neurons ($p < 0.02$, Tukey post hoc). Nevertheless, the absolute magnitude of preservation of neurons (31%) was less than anticipated, based on a much greater level of suppression of death in the acute phase.

a trend towards fewer apoptotic profiles in these sections among the CHOP null mice (2.7 ± 0.8 profiles/SN), this did not achieve significance in comparison with the wild-type (5.2 ± 1.1, $p > 0.1$, Tukey post hoc). We therefore attribute the marked difference in number of TH-positive neurons between the two genotypes to the well described suppression of TH phenotype following MPTP treatment (Jackson-Lewis *et al*. 1995). In keeping with this interpretation, in the chronic setting at 21 days post-lesion, there was only a 36% decrease in TH neuron number following MPTP in the wild-type animals. This apparent increase in the number of TH-positive neurons between the acute and chronic lesion periods has previously been shown to be due to a recovery of phenotype (Jackson-Lewis *et al*. 1995). In the chronic period, in the MPTP-treated mice, unlike the 6OHDA-treated mice, there was no protective effect of the null mutation on the number of surviving TH-positive neurons (Fig. 6c). This difference between the two models is in keeping with the lack of an effect of the null mutation on the magnitude of cell death in the acute period of the MPTP model, whereas there was a pronounced effect in the 6OHDA model. As would be expected from the lack of a protective effect of the null mutation on TH-positive neuron number, there was also no protective effect on striatal TH-positive fiber density (data not shown). We therefore conclude that in the chronic MPTP model, CHOP appears primarily to play a role in the loss of phenotype response that accompanies cellular injury, rather than in cell death, as it does in the 6OHDA model.

CHOP induction in neurotoxin models is not accompanied by changes in BiP mRNA expression, or the appearance of the XBP-1 splice variant

The induction of CHOP alone cannot be taken as compelling evidence for the occurrence of the ER stress response because CHOP can be induced by other cell stressors, such as oxidative stress, arsenite exposure and amino acid limitation (Bruhat *et al*. 1997; Jousse *et al*. 1999; Entingh *et al*. 2001; Mengesdorf *et al*. 2002). Therefore, to determine whether the induction of CHOP observed in these models was indicative of the broader ER stress response, we examined the mRNA expression of an ER-resident chaperone, BiP (also know as Grp78) (Gething 1999; Kaufman 1999). Induction of BiP mRNA has previously been shown to occur *in vitro* in conjunction with CHOP induction upon exposure of neuronal cells to 6OHDA (Ryu *et al*. 2002; Holtz and O'Malley 2003). In addition, we assessed nigral tissue by PCR for the presence of a splice variant of the transcription factor x-box binding protein-1 (XBP-1) (Yoshida *et al*. 2001; Calfon *et al*. 2002), a specific marker for the unfolded protein response.

Northern analysis of SN tissue from mice treated according to the chronic MPTP regimen on the last day of injection (PLD0) or 2 days after the final injection failed to demonstrate any change in BiP mRNA in comparison with saline-treated controls (not shown). To conduct an analysis of BiP mRNA expression at the SNpc regional and cellular levels in the adult 6OHDA model, we performed NRISH. As

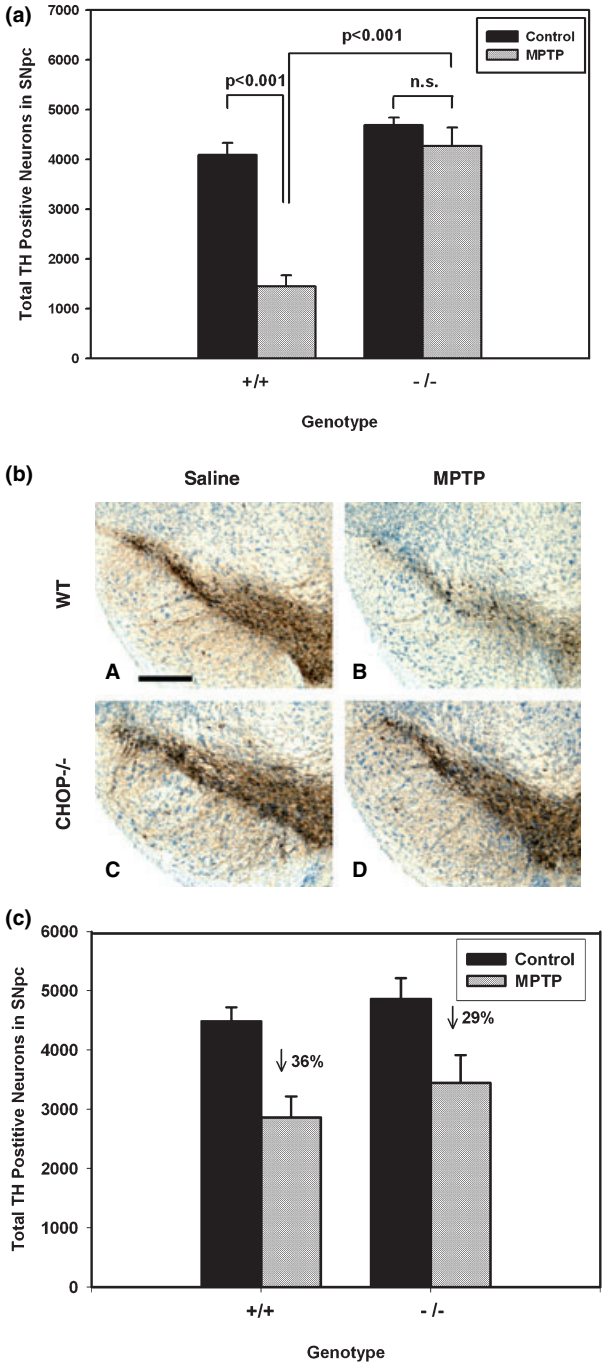

Fig. 6 The CHOP null mutation provides early protection from loss of phenotype, but not from neuron death, in the chronic MPTP model. (a) Wild-type and CHOP null adult mice were injected with saline or MPTP (30 mg/kg/day) for 5 days ($n = 4$ each group except wild-type saline, $n = 3$) and killed 4 days after the last dose for immunostaining and stereologic determination of TH-positive neuron number. Remarkably, there was minimal apparent effect in the CHOP nulls treated with MPTP. The wild-type mice showed a 65% decrease in number of TH-positive profiles. This difference could not be attributed to a change in the magnitude of apoptosis, as discussed in the text. (b) Representative low power photomicrographs demonstrating the resistance of SN dopamine neurons in CHOP null mice to the early effect (4 days post-lesion) of MPTP in the chronic injection model. These sections are derived from mice studied by stereologic analysis of TH-positive neuron number, shown in (a). Bar = 300 μm. (c) Wild-type and CHOP null mice were injected with saline or MPTP ($n = 4$–5 each group) and killed 21 days following the final injection for TH immunostaining and stereology. At this late post-lesion day, when the acute suppression of phenotype has recovered, it is apparent that there has been only a 36% loss of SN dopamine neurons in wild-type mice. While there was a trend for a reduction in the number of neurons lost in the CHOP null mice (29% loss), this did not achieve significance ($p > 0.5$, Tukey post-hoc).

previously reported by others for normal rat (Little *et al*. 1996), we observed widespread constitutive expression of BiP mRNA in brain (not shown). However, we did not observe any induction in SNpc, at the regional or cellular level, following unilateral intrastriatal 6OHDA injection at PLD2. A similar analysis of MPTP-treated mice failed to show any difference in BiP mRNA expression in SNpc in comparison with saline-treated controls (not shown).

Southern analysis of PCR reaction products for the XBP-1 unspliced and spliced variants was performed with the inclusion of a positive control derived from renal tissue of tunicamycin-treated mice, in which the ER stress response has previously been demonstrated (Zinszner *et al*. 1998). This analysis was performed for SN tissues derived from 6OHDA-treated mice at 1 and 3 days post-lesion, and for tissues derived from mice treated with MPTP according to both the acute and chronic regimens. In no instance was the XBP-1 splice variant identified in SN tissues, in spite of its clear presence in tunicamycin-treatment renal tissue. We conclude that in spite of the induction of CHOP protein in these models, there is no additional biochemical evidence of an unfolded protein response using these methods at the tissue level.

Discussion

The hypothesis that PCD plays a role in neural degeneration in PD rests principally on two forms of evidence. First, in rodent neurotoxin models, there is histological and biochemical evidence for activation of PCD mediators, such as the caspases, and functional evidence from genetic and pharmacological studies (reviewed in Vila and Przedborski 2003). Second, while traditional morphological assessments of human PD post-mortem brains for apoptosis have been controversial, there has been growing evidence for activation of caspases (Hartmann *et al*. 2000, 2001; Viswanath *et al*. 2001). While this evidence validates PCD as a target for the development of neuroprotective therapeutics, much remains unknown, particularly about upstream mediators that would make attractive therapeutic targets (Yuan and Yankner 2000).

The identification of CHOP as a markedly up-regulated transcript following the treatment of catecholaminergic cell lines with dopaminergic neurotoxins (Ryu *et al*. 2002; Holtz and O'Malley 2003) and with rotenone, a mitochondrial Complex 1 inhibitor (Ryu *et al*. 2002), is of particular interest because as a transcription factor, it would be likely to play an upstream regulatory role. In keeping with that possibility, a gene activated by CHOP, DOC6, is homologous to gelsolin, a mediator of cytoskeletal collapse during apoptosis (Wang *et al*. 1998). CHOP is also of particular interest in relation to PD because it has been implicated as an apoptotic mediator in the setting of oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002), which has been long postulated to play a role in PD (reviewed in Fahn and Cohen 1992), and ER stress (Matsumoto *et al*. 1996; Zinszner *et al*. 1998; Kawahara *et al*. 2001; Maytin *et al*. 2001; Gotoh *et al*. 2002; Oyadomari and Mori 2004), which has likewise recently been implicated in this disease (Imai *et al*. 2000, 2001).

We have determined that CHOP is expressed in neurotoxin animal models of parkinsonism. In a developmental model of apoptosis induced in dopamine neurons of the SN by the intrastriatal injection of 6OHDA (Marti *et al*. 1997), there was robust induction of CHOP protein expression exclusively within the SNpc. At a cellular level, CHOP expression was nuclear, as expected for a transcription factor, and exclusively within dopaminergic neurons. CHOP expression was also observed in neurotoxin models in the adult setting following intrastriatal 6OHDA, and either acute or chronic systemic MPTP exposure. In these adult models, as in the developmental 6OHDA model, CHOP expression was strictly within the SNpc at a regional level, and within the nucleus of otherwise normal-appearing neurons at a cellular level. CHOP expression, however, is not a universal feature of apoptosis in dopamine neurons; in the developmental setting, it is observed neither during naturally-occurring cell death (Janec and Burke 1993; Oo and Burke 1997), nor with augmentation of this death by axotomy (El-Khodor and Burke 2002). On the basis of classic neurotrophic theory (Clarke 1985), the naturally-occurring cell death event and its augmentation by axotomy would be considered to be regulated by the availability of neurotrophic support. Our observations that CHOP is not induced in these conditions, but it is by neurotoxic insults, are comparable with the *in vitro* observations of Ryu *et al*. (2002), who noted that CHOP is induced by neurotoxins, but not by neurotrophic withdrawal.

The principal finding of these investigations was that adult CHOP null mice were resistant to apoptotic death in SN dopamine neurons induced by the intrastriatal injection of 6OHDA. We considered the possibilities that this reduction may be due to a change in the time course of apoptosis, or to the rate of clearance of apoptotic profiles in the null mice, rather than an actual reduction in the eventual magnitude of the death event. We therefore assessed the final surviving number of SN DA neurons at PLD28 and found that they were increased, indicating that the null mutation did in fact reduce the magnitude of death. We therefore conclude that CHOP is an important functional mediator of apoptosis in the 6OHDA model. Given that CHOP is highly expressed prior to any morphologic change in dopamine neurons destined to die in this model, we postulate that CHOP is likely to be an early mediator in the death process. Although the CHOP null mutation was protective in this model, the degree of preservation of SN dopamine neurons in absolute terms, 31%, was less than anticipated based on a 65% suppression of apoptotic death in the early post-lesion period. This discrepancy suggests that some of the death which ultimately occurs in the CHOP nulls is delayed. There are two possible explanations for this delay. First, death mediators other than CHOP may eventually come into play (Ryu *et al*. 2005) and bring about the loss of the majority of dopamine neurons. Second, in these non-temporally-regulated nulls, compensatory changes may have taken place to provide alternate death pathways. These two possibilities are not mutually exclusive.

In view of the ability of the CHOP null mutation to provide neuroprotection in the adult 6OHDA model, the question arises as to why it did not also provide protection in the postnatal model, in which CHOP expression is clearly induced. Our interpretation of this difference rests on our previous demonstrations that during the first two postnatal weeks, SN dopamine neurons are dependent on interactions with their target, the striatum, as envisioned by classic neurotrophic theory (Clarke 1985), whereas in adults they are not (Macaya *et al*. 1994; Kelly and Burke 1996; Stefanis and Burke 1996). Therefore, during this postnatal period, the death of SN dopamine neurons following the destruction of their nerve terminals with 6OHDA is likely to be mediated by an 'axotomy' effect as well as a direct neurotoxic effect. This interpretation is supported not only by the aforementioned studies of the developmental time course of striatal target dependence, but also by our demonstrations that the postnatal 6OHDA model is characterized by two cellular patterns of caspase activation: a perinuclear pattern, as observed in naturally-occurring cell death (Jeon *et al*. 1999; El-Khodor and Burke 2002; Oo *et al*. 2002), and a cytoplasmic pattern, observed in direct neurotoxic injury (Jeon *et al*. 1999; Oo *et al*. 2002). Given this likelihood of an axotomy effect in the postnatal 6OHDA model, and based on our demonstration herein that CHOP is not expressed following developmental axotomy, we would anticipate that a functional role for CHOP would be difficult to discern in the postnatal 6OHDA lesion.

MPP^+, the toxic metabolite of MPTP, induced CHOP expression in *in vitro* models (Ryu *et al*. 2002; Holtz and O'Malley 2003). MPTP treatment *in vivo* likewise induced the expression of CHOP protein, but in the chronic MPTP

model, unlike the 6OHDA model, the CHOP null mutation did not significantly diminish the level of apoptosis or increase the number of surviving neurons. The null mutation did, however, prevent the loss of TH immunoreactivity in the period early after the MPTP injections. We interpret this relative preservation of TH immunoreactivity in the absence of protection from cell death to be attributable to protection from the loss of phenotype, which is well documented in this (Jackson-Lewis *et al*. 1995) and other neuronal injury models (Wooten *et al*. 1978). We conclude that while CHOP plays a role in regulating cellular phenotype in the MPTP model, it is not likely to play a role as an important death mediator. This difference in the role of CHOP between the 6OHDA and MPTP models in living animals is consistent with the observations made *in vitro* by Holtz and O'Malley (2003). Following treatment with 6OHDA, they observed a greater induction of CHOP and a more general induction of other ER stress markers than with MPTP treatment.

To determine whether the CHOP induction observed in these neurotoxin models was specifically due to ER stress, we assayed mRNA expression of the ER-resident chaperone BiP (Gething 1999; Kaufman 1999) and the splice variant of XBP-1 (Yoshida *et al*. 2001; Calfon *et al*. 2002). In none of the models was there a change observed in BiP mRNA expression or the appearance of the XBP-1 splice variant. These results were not unexpected for the MPTP model in view of *in vitro* results that showed no induction of BiP or XBP-1 by MPP^+ (Holtz and O'Malley 2003). However, the results were unexpected for the 6OHDA model as both prior *in vitro* studies had demonstrated clear evidence for a full ER stress response induced by 6OHDA (Ryu *et al*. 2002; Holtz and O'Malley 2003). There are two principal interpretations of these negative results. First, it is possible that CHOP induction in the 6OHDA model in living animals is not part of a full ER stress response, the *in vitro* results notwithstanding. It is well established that 6OHDA produces oxidative stress (Heikkila and Cohen 1973; Cohen and Heikkila 1974). It is therefore possible that its induction of CHOP in living animals is mediated principally by cellular oxidative stress (Guyton *et al*. 1996; Mengesdorf *et al*. 2002). Alternatively, it is possible that the studies of BiP and the XBP-1 splice variant that were performed at the tissue level lacked the sensitivity to detect changes, which, for CHOP, were detected at the cellular level by immunohistochemistry. Thus, our inability to detect other markers for ER stress in the 6OHDA model does not permit us to definitively conclude that it is not present.

We conclude that these investigations performed in living animals are largely supportive of the *in vitro* results suggesting the possibility of a role for CHOP in the neurodegeneration associated with PD. We find, as predicted from these gene expression screens, that CHOP is expressed in diverse neurotoxin models of dopamine neuron death. These observations support the validity of the *in vitro* screens for genes of potential relevance to disease. In addition, we find that CHOP can play a role as a mediator of cell death, depending on the context; in the 6OHDA model, CHOP is a necessary death mediator. The context specificity of CHOP is an important feature, because it suggests that it may be possible in designing neuroprotection strategies to target disease-related death pathways without interfering with other apoptotic pathways that may be important for survival of the organism.

Acknowledgements

This work was supported by NS26836, NS38370, DAMD17-03-1-0492, ES08681, NS43628, The Parkinson's Disease Foundation and the Michael J. Fox Foundation. We gratefully acknowledge the quantitative morphological analysis performed by Ms Rebecca Greene.

References

Bruhat A., Jousse C., Wang X. Z., Ron D., Ferrara M. and Fafournoux P. (1997) Amino acid limitation induces expression of CHOP, a CCAAT/enhancer binding protein-related gene, at both transcriptional and post-transcriptional levels. *J. Biol. Chem.* **272**, 17 588–17 593.

Burke R. E., Franklin S. O. and Inturrisi C. E. (1994) Acute and persistent suppression of preproenkephalin mRNA expression in the striatum following developmental hypoxic-ischemic injury. *J. Neurochem.* **62**, 1878–1886.

Calfon M., Zeng H., Urano F., Till J. H., Hubbard S. R., Harding H. P., Clark S. G. and Ron D. (2002) IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. *Nature* **415**, 92–96.

Ciechanover A. (1998) The ubiquitin-proteasome pathway: on protein death and cell life. *EMBO J.* **17**, 7151–7160.

Clarke P. G. H. (1985) Neuronal death in the development of the vertebrate nervous system. *Trends Neurosci.* **8**, 345–349.

Coggeshall R. E. and Lekan H. A. (1996) Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J. Comp. Neurol.* **364**, 6–15.

Cohen G. and Heikkila R. E. (1974) The generation of hydrogen peroxide, superoxide radical, and hydroxyl radical by 6-hydroxydopamine, dialuric acid, and related cytotoxic agents. *J. Biol. Chem.* **249**, 2447–2452.

El-Khodor B. F. and Burke R. E. (2002) Medial forebrain bundle axotomy during development induces apoptosis in dopamine neurons of the substantia nigra and activation of caspases in their degenerating axons. *J. Comp. Neurol.* **452**, 65–79.

El-Khodor B. F., Kholodilov N. G., Yarygina O. and Burke R. E. (2001) The expression of mRNAs for the proteasome complex is developmentally regulated in the rat mesencephalon. *Brain Res. Dev. Brain Res.* **129**, 47–56.

Entingh A. J., Law B. K. and Moses H. L. (2001) Induction of the C/EBP homologous protein (CHOP) by amino acid deprivation requires insulin-like growth factor I, phosphatidylinositol 3-kinase, and mammalian target of rapamycin signaling. *Endocrinology* **142**, 221–228.

Fahn S. and Cohen G. (1992) The oxidant stress hypothesis in Parkinson's disease: Evidence supporting it. *Ann. Neurol.* **32**, 804–812.

Ganguly A., Oo T. F., Rzhetskaya M., Pratt R., Yarygina O., Momoi T., Kholodilov N. and Burke R. E. (2004) CEP11004, a novel inhibitor of the mixed lineage kinases, suppresses apoptotic death in

dopamine neurons of the substantia nigra induced by 6-hydroxydopamine. *J. Neurochem.* **88**, 469–480.

Gething M. J. (1999) Role and regulation of the ER chaperone BiP. *Semin. Cell Dev. Biol.* **10**, 465–472.

Gotoh T., Oyadomari S., Mori K. and Mori M. (2002) Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J. Biol. Chem.* **277**, 12 343–12 350.

Greene L. A. and Tischler A. S. (1976) Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl Acad. Sci. USA* **73**, 2424–2428.

Guyton K. Z., Xu Q. and Holbrook N. J. (1996) Induction of the mammalian stress response gene GADD153 by oxidative stress: role of AP-1 element. *Biochem. J.* **314**, 547–554.

Hartmann A., Hunot S., Michel P. P. *et al.* (2000) Caspase-3: a vulnerability factor and final effector in apoptotic death of dopaminergic neurons in Parkinson's disease. *Proc. Natl Acad. Sci. USA* **97**, 2875–2880.

Hartmann A., Troadec J. D., Hunot S., Kikly K., Faucheux B. A., Mouatt-Prigent A., Ruberg M., Agid Y. and Hirsch E. C. (2001) Caspase-8 is an effector in apoptotic death of dopaminergic neurons in Parkinson's disease, but pathway inhibition results in neuronal necrosis. *J. Neurosci.* **21**, 2247–2255.

Heikkila R. E. and Cohen G. (1973) 6-Hydroxydopamine: evidence for superoxide radical as an oxidative intermediate. *Science* **181**, 456–457.

Heikkila R. E., Hess A. and Duvoisin R. C. (1984) Dopaminergic neurotoxicity of 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine in mice. *Science* **224**, 1451–1453.

Heller A., Hutchens J. O., Kirby M. L., Karapas F. and Fernandez C. (1979) Stereotaxic electrode placement in the neonatal rat. *J. Neurosci. Meth.* **1**, 41–76.

Holtz W. A. and O'Malley K. L. (2003) Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J. Biol. Chem.* **278**, 19 367–19 377.

Imai Y., Soda M. and Takahashi R. (2000) Parkin suppresses unfolded protein stress-induced cell death through its E3 ubiquitin-protein ligase activity. *J. Biol. Chem.* **275**, 35 661–35 664.

Imai Y., Soda M., Inoue H., Hattori N., Mizuno Y. and Takahashi R. (2001) An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. *Cell* **105**, 891–902.

Immanuel D., Zinszner H. and Ron D. (1995) Association of SARFH (sarcoma-associated RNA-binding fly homolog) with regions of chromatin transcribed by RNA polymerase II. *Mol. Cell Biol.* **15**, 4562–4571.

Ishikawa A. and Tsuji S. (1996) Clinical analysis of 17 patients in 12 Japanese families with autosomal-recessive type juvenile parkinsonism. *Neurology* **47**, 160–166.

Jackson-Lewis V., Jakowec M., Burke R. E. and Przedborski S. (1995) Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine. *Neurodegeneration* **4**, 257–269.

Janec E. and Burke R. E. (1993) Naturally occurring cell death during postnatal development of the substantia nigra of the rat. *Mol. Cell Neurosci.* **4**, 30–35.

Jeon B. S., Kholodilov N. G., Oo T. F., Kim S., Tomaselli K. J., Srinivasan A., Stefanis L. and Burke R. E. (1999) Activation of caspase-3 in developmental models of programmed cell death in neurons of the substantia nigra. *J. Neurochem.* **73**, 322–333.

Jousse C., Bruhat A., Harding H. P., Ferrara M., Ron D. and Fafournoux P. (1999) Amino acid limitation regulates CHOP expression through a specific pathway independent of the unfolded protein response. *FEBS Lett.* **448**, 211–216.

Kaufman R. J. (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. *Genes Dev.* **13**, 1211–1233.

Kawahara K., Oyadomari S., Gotoh T., Kohsaka S., Nakayama H. and Mori M. (2001) Induction of CHOP and apoptosis by nitric oxide in p53-deficient microglial cells. *FEBS Lett.* **506**, 135–139.

Kelly W. J. and Burke R. E. (1996) Apoptotic neuron death in rat substantia nigra induced by striatal excitotoxic injury is developmentally dependent. *Neurosci. Lett.* **220**, 85–88.

Kitada T., Asakawa S., Hattori N., Matsumine H., Yamamura Y., Minoshima S., Yokochi M., Mizuno Y. and Shimizu N. (1998) Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392**, 605–608.

Kostrzewa R. M. and Jacobowitz D. M. (1974) Pharmacological actions of 6-hydroxydopamine. *Pharmacol. Rev.* **26**, 199–288.

Little E., Tocco G., Baudry M., Lee A. S. and Schreiber S. S. (1996) Induction of glucose-regulated protein (glucose-regulated protein 78/BiP and glucose-regulated protein 94) and heat shock protein 70 transcripts in the immature rat brain following status epilepticus. *Neuroscience* **75**, 209–219.

Macaya A., Munell F., Gubits R. M. and Burke R. E. (1994) Apoptosis in substantia nigra following developmental striatal excitotoxic injury. *Proc. Natl Acad. Sci. USA* **91**, 8117–8121.

Marti M. J., James C. J., Oo T. F., Kelly W. J. and Burke R. E. (1997) Early developmental destruction of terminals in the striatal target induces apoptosis in dopamine neurons of the substantia nigra. *J. Neurosci.* **17**, 2030–2039.

Martin D. P., Schmidt R. E., DiStefano P., Lowry O., Carter J. and Johnson E. (1988) Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J. Cell Biol.* **106**, 829–844.

Matsumoto M., Minami M., Takeda K., Sakao Y. and Akira S. (1996) Ectopic expression of CHOP (GADD153) induces apoptosis in M1 myeloblastic leukemia cells. *FEBS Lett.* **395**, 143–147.

Mattson M. P. (2000) Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* **1**, 120–129.

Maytin E. V., Ubeda M., Lin J. C. and Habener J. F. (2001) Stress-inducible transcription factor CHOP/gadd153 induces apoptosis in mammalian cells via p38 kinase-dependent and -independent mechanisms. *Exp. Cell Res.* **267**, 193–204.

Mengesdorf T., Althausen S. and Paschen W. (2002) Genes associated with pro-apoptotic and protective mechanisms are affected differently on exposure of neuronal cell cultures to arsenite. No indication for endoplasmic reticulum stress despite activation of grp78 and gadd153 expression. *Brain Res. Mol. Brain Res.* **104**, 227–239.

Oo T. F. and Burke R. E. (1997) The time course of developmental cell death in phenotypically defined dopaminergic neurons of the substantia nigra. *Dev. Brain Res.* **98**, 191–196.

Oo T. F., Siman R. and Burke R. E. (2002) Distinct nuclear and cytoplasmic localization of caspase cleavage products in two models of induced apoptotic death in dopamine neurons of the substantia nigra. *Exp. Neurol.* **175**, 1–9.

Oo T. F., Kholodilov N. and Burke R. E. (2003) Regulation of natural cell death in dopaminergic neurons of the substantia nigra by striatal GDNF *in vivo*. *J. Neurosci.* **23**, 5141–5148.

Oppenheim R. W. (1991) Cell death during development of the nervous system. *Ann. Rev. Neurosci.* **14**, 453–501.

Oppenheim R. W., Prevette D., Tytell M. and Homma S. (1990) Naturally occurring and induced neuronal death in the chick embryo *in vivo* requires protein and RNA synthesis: Evidence for the role of cell death genes. *Dev. Biol.* **138**, 104–113.

Oyadomari S. and Mori M. (2004) Roles of CHOP/GADD153 in endoplasmic reticulum stress. *Cell Death Differ.* **11**, 381–389.

Przedborski S. and Vila M. (2003) The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. *Ann. N Y Acad. Sci.* **991**, 189–198.

Przedborski S., Jackson-Lewis V., Naini A. B., Jakowec M., Petzinger G., Miller R. and Akram M. (2001) The parkinsonian toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP): a technical review of its utility and safety. *J. Neurochem.* **76**, 1265–1274.

Ron D. and Habener J. F. (1992) CHOP, a novel developmentally regulated nuclear protein that dimerizes with transcription factors C/EBP and LAP and functions as a dominant-negative inhibitor of gene transcription. *Genes Dev.* **6**, 439–453.

Ryu E. J., Harding H. P., Angelastro J. M., Vitolo O. V., Ron D. and Greene L. A. (2002) Endoplasmic reticulum stress and the unfolded protein response in cellular models of Parkinson's disease. *J. Neurosci.* **22**, 10 690–10 698.

Ryu E. J., Angelastro J. M. and Greene L. A. (2005) Analysis of gene expression changes in a cellular model of Parkinson disease. *Neurobiol. Dis.* **18**, 54–74.

Sauer H. and Oertel W. H. (1994) Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6 hydroxydopamine a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience* **59**, 401–415.

Senoh S. and Witkop B. (1959) Formation and rearrangements of aminochromes from a new metabolite of dopamine and some of its derivatives. *J. Am. Chem. Soc.* **81**, 6231–6235.

Shimura H., Hattori N., Kubo S. *et al.* (2000) Familial parkinson disease gene product, parkin, is a ubiquitin-protein ligase. *Nat. Genet.* **25**, 302–305.

Stefanis L. and Burke R. E. (1996) Transneuronal degeneration in substantia nigra pars reticulata following striatal excitotoxic injury in adult rat: Time course, distribution, and morphology of cell death. *Neuroscience* **74**, 997–1008.

Tatton N. A. and Kish S. J. (1997) *In situ* detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labelling and acridine orange. *Neuroscience* **77**, 1037–1048.

Teismann P., Tieu K., Choi D. K., Wu D. C., Naini A., Hunot S., Vila M., Jackson-Lewis V. and Przedborski S. (2003) Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. *Proc. Natl Acad. Sci. USA* **100**, 5473–5478.

Vila M. and Przedborski S. (2003) Targeting programmed cell death in neurodegenerative diseases. *Nat. Rev. Neurosci.* **4**, 365–375.

Viswanath V., Wu Y., Boonplueang R., Chen S., Stevenson F. F., Yantiri F., Yang L., Beal M. F. and Andersen J. K. (2001) Caspase-9 activation results in downstream caspase-8 activation and bid cleavage in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced Parkinson's disease. *J. Neurosci.* **21**, 9519–9528.

Wang X. Z., Kuroda M., Sok J., Batchvarova N., Kimmel R., Chung P., Zinszner H. and Ron D. (1998) Identification of novel stress-induced genes downstream of chop. *EMBO J.* **17**, 3619–3630.

Wooten G. F., Park D. H., Joh T. H. and Reis D. J. (1978) Immunochemical demonstration of reversible reduction in choline acetyltransferase concentration in rat hypoglossal nucleus after hypoglossal nerve transection. *Nature* **275**, 324–325.

Yoshida H., Matsui T., Yamamoto A., Okada T. and Mori K. (2001) XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. *Cell* **107**, 881–891.

Yuan J. and Yankner B. A. (2000) Apoptosis in the nervous system. *Nature* **407**, 802–809.

Zinszner H., Kuroda M., Wang X., Batchvarova N., Lightfoot R. T., Remotti H., Stevens J. L. and Ron D. (1998) CHOP is implicated in programmed cell death in response to impaired function of the endoplasmic reticulum. *Genes Dev.* **12**, 982–995.

Oncoprotein Akt/PKB induces trophic effects in murine models of Parkinson's disease

Vincent Ries*, Claire Henchcliffe*, Tatyana Kareva*, Margarita Rzhetskaya*, Ross Bland†, Matthew J. During‡, Nikolai Kholodilov*, and Robert E. Burke*§¶

Departments of *Neurology and §Pathology, Columbia University College of Physicians and Surgeons, New York, NY 10032; and †Neurologix Research and ‡Human Cancer Genetics Program, Ohio State University Comprehensive Cancer Center, Columbus, OH 43210

Edited by Solomon H. Snyder, Johns Hopkins University School of Medicine, Baltimore, MD, and approved October 11, 2006 (received for review July 27, 2006)

Despite promising preclinical studies, neurotrophic factors have not yet achieved an established role in the treatment of human neurodegenerative diseases. One impediment has been the difficulty in providing these macromolecules in sufficient quantity and duration at affected sites. An alternative approach is to directly activate, by viral vector transduction, intracellular signaling pathways that mediate neurotrophic effects. We have evaluated this approach in dopamine neurons of the substantia nigra, neurons affected in Parkinson's disease, by adeno-associated virus 1 transduction with a gene encoding a myristoylated, constitutively active form of the oncoprotein Akt/PKB. Adeno-associated virus Myr-Akt has pronounced trophic effects on dopamine neurons of adult and aged mice, including increases in neuron size, phenotypic markers, and sprouting. Transduction confers almost complete protection against apoptotic cell death in a highly destructive neurotoxin model. Activation of intracellular neurotrophic signaling pathways by vector transfer is a feasible approach to neuroprotection and restorative treatment of neurodegenerative disease.

apoptosis | dopamine | neurotrophic | programmed cell death | substantia nigra

Parkinson's disease (PD) is a major public health problem in the world, with an estimated 1,000,000 individuals affected in the United States alone (1). It is a chronic, progressive neurodegenerative disorder that typically manifests with impairments of motor function, including tremor, rigidity, slowness of movement, and poor postural stability, attributable to the loss of dopamine (DA) neurons of the substantia nigra (SN) of the midbrain. These motor manifestations can be treated successfully for a limited period, either with drugs, which restore dopaminergic function, or more recently by deep brain stimulation. However, there is no treatment that forestalls deterioration attributable to progressive neurodegeneration.

For neurodegenerative diseases, one approach that has held promise for providing both neuroprotection and restoration is the administration of protein neurotrophic factors. In PD, substantial effort has been made to explore the possibility of providing neuroprotection and restoration by the intracerebral administration of glial cell line-derived neurotrophic factor (GDNF) (2). A pilot study of intracerebral infusion of GDNF offered promise (3), but a subsequent, larger, double-blind trial failed to demonstrate benefit (4). Although there are many possible reasons for this discrepancy, it is likely that the technical difficulties inherent in reliably providing sufficient quantities of neurotrophic protein within brain parenchyma of affected regions plays a role. These technical constraints apply to the use of other neurotrophic factors in other neurodegenerative diseases as well (5).

An alternative to delivering neurotrophic protein molecules within brain extracellular space is to directly activate the intracellular signaling pathways responsible for their effects. This activation is possible by viral vector approaches to transduction of neurons (6). One such intracellular pathway utilizes the phosphatidylinositide 3′-OH kinase and Akt/PKB signaling cascade (7, 8). In neurons, Akt activation has been identified in response to treatment with insulin-like growth factor 1 (9), nerve growth factor (10), and glial cell line-derived neurotrophic factor (11). Akt signaling mediates two principal cellular responses to neurotrophic factors. It maintains viability through antiapoptotic effects [reviewed in Brunet *et al*. (12)], and it mediates effects on axonal caliber, branching (13), and regeneration (14). Almost all of these observations, however, have been made *in vitro*, and it has not been known whether they generalize to the *in vivo* context, particularly in the central nervous system. We therefore have examined whether Akt mediates trophic effects on adult normal, neurotoxin-lesioned, and aged DA neurons of the SN by adeno-associated virus (AAV)-mediated transduction with a myristoylated (Myr), constitutively active form (15).

Results

Expression of Endogenous Akt Isoforms in SN. We examined expression of endogenous Akt mRNA and protein in the SN (Fig. 5, which is published as supporting information on the PNAS web site). Of the three Akt isoforms, Akt1 mRNA was the most abundantly expressed in SN. At a regional level, Akt1 mRNA was most highly expressed in the SN pars compacta (SNpc), and, at a cellular level, it was expressed exclusively within neurons (data not shown). The same was true for the Akt2 and Akt3 isoforms. The developmental pattern of expression of Akt1 mRNA within SN differed from that observed in striatum and cortex, in that it was relatively sustained in adulthood, at levels 80% of those at postnatal day (PND) 2 (Fig. 5*C*). In striatum and cortex, mRNA levels in adulthood were ≈40% of those at PND2. At the protein level, phospho-Akt(Ser-473) was detected within the SNpc and specifically within DA neurons (Fig. 5*D*).

Trophic Effects of AAV Myr-Akt in Normal Adult Mice. Transduction of neurons of the SN of adult male mice with AAV Myr-Akt induced a 50% increase in nigral volume (Fig. 1*A*). At a cellular level, for the SNpc, this increased regional volume was attributable, in part, to a 50% increase in neuron size (Fig. 1*B*). There was, in addition, a 44% increase in the number of tyrosine hydroxylase (TH)-positive neurons (Fig. 1*C*). We attribute this

Author contributions: V.R., C.H., M.J.D., N.K., and R.E.B. designed research; V.R., C.H., T.K., M.R., R.B., and N.K. performed research; V.R., C.H., and R.E.B. analyzed data; and V.R., C.H., R.B., M.J.D., N.K., and R.E.B. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

Freely available online through the PNAS open access option.

Abbreviations: AAV, adeno-associated virus; PD, Parkinson's disease; DA, dopamine; SN, substantia nigra; Myr, myristoylated; SNpc, SN pars compacta; TH, tyrosine hydroxylase; HVA, homovanillic acid; 6OHDA, 6-hydroxydopamine.

¶To whom correspondence should be addressed at: Department of Neurology, Room 306, Black Building, Columbia University Medical Center, 650 West 168th Street, New York, NY 10032. E-mail: rb43@columbia.edu.

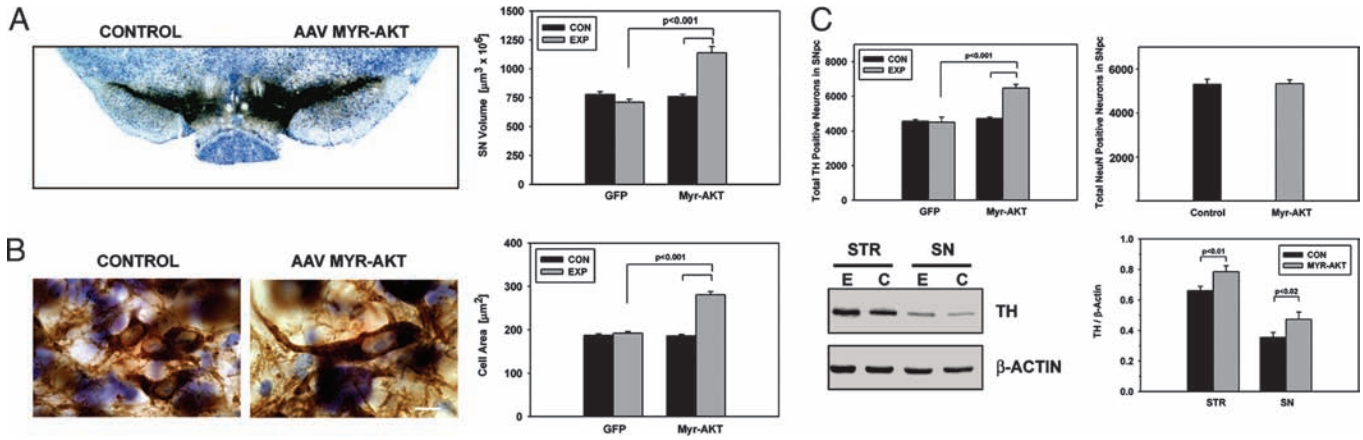

Fig. 1. Trophic effects of Myr-Akt on DA neurons of the SN in adult mice. (*A*) A section processed for TH immunostaining and thionin counterstain at 7 weeks after AAV Myr-Akt injection demonstrates an increase in the size of the SN and the SNpc. The volume of the injected (EXP, experimental) SN was 1,139.2 ± 54.1 ($\times 10^6$) μm^3 (mean ± SEM), a 50% increase in comparison to the contralateral, noninjected control (CON) side (760.6 ± 18.5 ($\times 10^6$) μm^3) ($P < 0.001$; $n = 6$ animals for Myr-Akt and GFP groups). (*B*) At a cellular level, the increased volume of the SNpc was attributable, at least in part, to an increase in the size of DA neurons. (Bar: 10 μm.) The cross-sectional areas of the TH-positive neurons in the SNpc of Myr-Akt-injected mice was 281.2 ± 7.1 μm^2 (mean ± SEM), a 50% increase in comparison to the contralateral, noninjected control side (185.5 ± 3.3 μm^2) ($P < 0.001$; $n = 2$ mice for both GFP control and Myr-Akt; $n = 50$ neurons in all conditions). (*C*) The increase in the volume of the SNpc also was accompanied by an increase in the number of TH-positive neurons. In the AAV Myr-Akt-injected SN, there was a mean of 6,483 ± 204 neurons, a 44% increase in comparison to the AAV GFP-injected SN (4,504 ± 295) ($P < 0.001$, ANOVA; $n = 6$ animals in both groups). Counts of total SNpc neurons, determined as stereologic counts of NeuN-stained profiles were unchanged (*Right*) ($n = 4$ animals). An increased level of TH expression in the SN after AAV Myr-Akt was demonstrated by Western analysis, as shown in *Lower Left*, and in a quantitative analysis of TH band optical densities normalized for β-actin ($n = 5$ animals). An increase in TH protein expression also was observed in striatum (STR) (E, experimental, AAV Myr-Akt-injected side; C, control, contralateral side).

apparent increase in DA neuron number to an increased level of expression of TH, determined by Western blotting analysis (Fig. 1*C*), and a resulting increase in detection by immunostaining, rather than an actual increase in the number of neurons, because counts of neuronal profiles in the SNpc, detected by NeuN immunostaining, were unchanged (Fig. 1*C*). These morphologic changes were accompanied by changes in the levels of DA (137% increase above AAV GFP control), and its metabolites homovanillic acid (HVA) (148% increase) and 3,4-dihydroxyphenylacetic acid (DOPAC) (35% increase) (Fig. 6, which is pub-

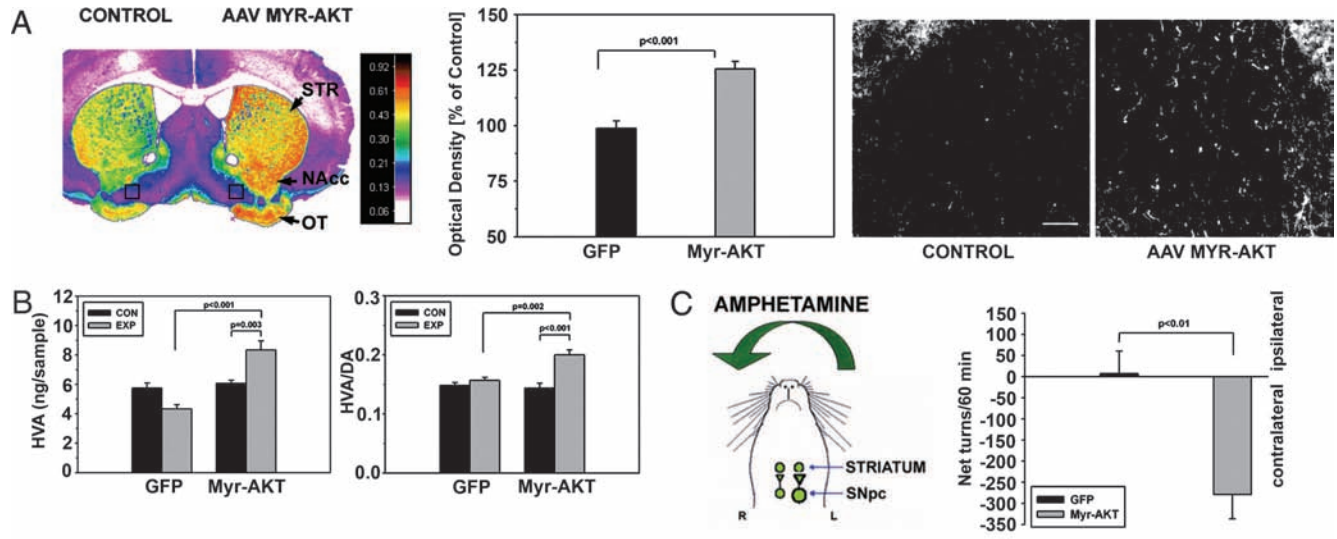

Fig. 2. Trophic effects of Myr-Akt on the axonal projections of SN DA neurons. (*A*) (*Left*) A pseudocolor image of a coronal section through the striatum, processed for TH immunostaining at 7 weeks after intranigral AAV Myr-Akt injection, reveals higher density values (red-orange) in the striatum (STR) on the injected side. Higher-density values also are observed in the nucleus accumbens (NAcc) and olfactory tubercle (OT). (*Center*) An increase in the optical density of the striatal TH immunostaining is shown quantitatively as a 26% increase over values for the contralateral, noninjected striatum; this was a highly significant increase in comparison to AAV GFP-injected animals ($P < 0.001$, *t* test; $n = 6$ animals for both groups). (*Right*) At a cellular level, this increase in optical density was attributable to an increase in the number and caliber of TH-positive fibers. (Bar: 50 μm.) The regions shown in these micrographs are between the accumbens and olfactory tubercle on both sides of the section (indicated as black rectangles in *Left*). (*B*) These morphologic changes were accompanied by biochemical changes indicative of increased DA release in the striatum. There was an increase in both HVA ($P < 0.001$) and the HVA/DA ratio ($P = 0.002$, ANOVA, AAV Myr-Akt-injected side compared with AAV GFP-injected side; $n = 6$ animals in each group) (EXP, experimental, injected side; CON, control, noninjected side). (*C*) (*Left*) When AAV Myr-Akt-injected mice were administered amphetamine (5 mg/kg i.p.), they exhibited rotational behavior contraversive to the side of the vector injection. (*Right*) Contraversive rotations are plotted as negative net rotations. AAV GFP-injected mice did not demonstrate such behavior.

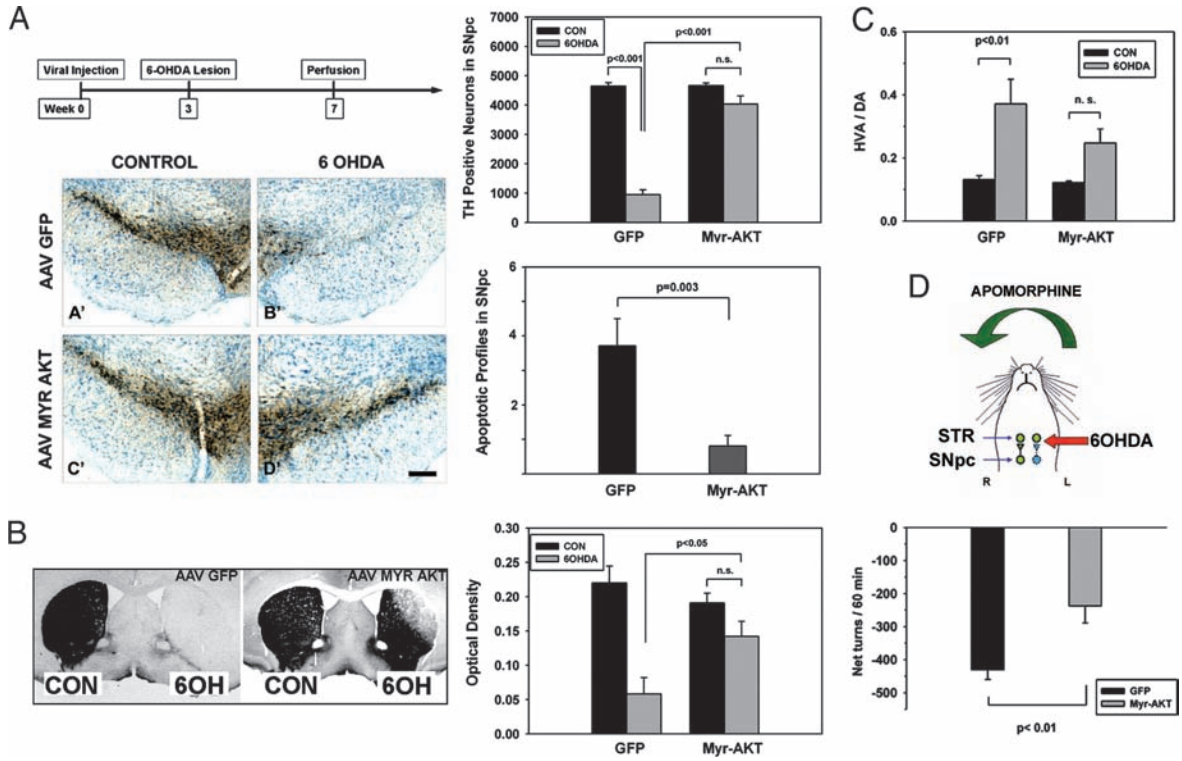

Fig. 3. Neuroprotective effects of Myr-Akt in the intrastriatal 6OHDA mouse model. (*A*) (*Left Upper*) The chronic morphologic studies shown in *A* and *B* were performed according to the timeline shown. The studies of apoptosis were conducted at 6 days after 6OHDA. (*Left Lower*) Mice treated with an AAV GFP control injection show an almost complete loss of TH-positive SNpc neurons. This loss is almost completely prevented in mice injected with AAV Myr-Akt (*D'*). (Bar: 200 μm.) (*Right Upper*) This effect is shown quantitatively as stereologic counts of the number of surviving TH-positive neurons. In mice given AAV GFP, TH-positive neuron numbers were reduced to 950 $\pm$ 163 after 6OHDA injection, or 20% of the noninjected contralateral control (CON) (4,646 $\pm$ 119). In mice given AAV Myr-Akt, neuron numbers were reduced to only 4,036 $\pm$ 280, or 87% of the contralateral control (4,664 $\pm$ 85). The difference between the AAV GFP and AAV Myr-Akt groups was highly significant ($P < 0.001$, ANOVA; $n = 6$ animals GFP; $n = 7$ animals Myr-Akt). (*Right Lower*) This protective effect of Myr-Akt on SNpc DA neurons was attributable to suppression of apoptosis as shown. The number of apoptotic profiles in the SNpc was reduced to 22% of the number observed in AAV GFP-treated mice ($P = 0.003$, *t* test; $n = 7$ animals AAV GFP; $n = 8$ animals AAV Myr-Akt). (*B*) The neuroprotective effect of Myr-Akt was observed at the level of the striatal axonal projections as well. (*Left*) In control AAV GFP-injected mice, there is an almost complete loss of striatal TH-positive staining on the 6OHDA-injected side. In AAV Myr-Akt-injected mice, there is some loss of immunoperoxidase labeling in the dorsolateral quadrant of the injected striatum, but otherwise staining of TH fibers is preserved. The mice used for this analysis were the same as those used for the stereologic analysis in *A*. (*Right*) The protective effect of Myr-Akt on striatal TH-positive fibers is shown quantitatively as optical densities of TH staining. (*C*) The morphologic preservation of striatal dopaminergic fibers was accompanied by relative preservation of biochemical indices of dopaminergic terminals. At 4 weeks after 6OHDA, striatal DA levels in AAV Myr-Akt-treated mice were 19.8 $\pm$ 4.2 ng per sample, a 3.4-fold increase over the mean value of 5.8 $\pm$ 2.9 observed in AAV GFP-treated mice ($P < 0.03$; $n =$ 6 animals AAV GFP; $n = 8$ animals AAV Myr-Akt) (data not shown). HVA levels were increased by 3-fold (data not shown). After lesions of the nigrostriatal dopaminergic projection, there is a compensatory increase in DA turnover, reflected in an increased HVA/DA ratio (16). This effect was observed in AAV GFP-treated mice as an increase in the HVA/DA ratio from 0.13 $\pm$ 0.01 in the nonlesioned striatum to 0.37 $\pm$ 0.08 in the lesioned striatum ($P = 0.006$). However, in the AAV Myr-Akt-treated mice, although there was a trend for this effect, it did not achieve significance. (*D*) To assess functionality of the nigrostriatal projection we examined apomorphine-induced rotations. After partial destruction of the dopaminergic projection, postsynaptic supersensitivity to direct-acting DA agonists, such as apomorphine, results in contraversive rotations (29). Strong contraversive rotational behavior was observed in AAV GFP-treated mice after apomorphine injection (0.5 mg/kg) at 4 weeks after 6OHDA lesion. This contraversive rotational behavior was significantly diminished in mice treated with AAV Myr-Akt (AAV GFP, -431 ± 29 net turns per 60 min; AAV Myr-Akt, -237 ± 52; $P = 0.006$, *t* test; $n = 7$ animals GFP; $n = 6$ animals Myr-Akt).

lished as supporting information on the PNAS web site). The increase in neuron size was not confined to DA neurons. Nondopaminergic neurons of the SN pars reticulata also were increased by 76% (control, noninjected SNpr: 177 $\pm$ 7 μm^2; AAV Myr-Akt: 312 $\pm$ 13 μm^2; $n = 2$ mice; 40 neurons total; $P <$ 0.001).

These trophic effects at the level of DA neuron cell bodies were accompanied by effects in their axonal projections within the striatum. At 7 weeks after intranigral injection of AAV Myr-Akt, there was an increased density of TH-immunoreactive fibers within the ipsilateral striatum (Fig. 2*A*). At a cellular level, although some of this increase in staining density could be attributed to an induction of TH protein expression (Fig. 1*C*), there also was a clear induction of fiber sprouting (Fig. 2*A*). In addition, the caliber of fibers was increased by 37% (contralateral control: 1.6 $\pm$ 0.1 μm; AAV Myr-Akt: 2.2 $\pm$ 0.1 μm; $P <$ 0.001; paired *t* test; $n = 3$ mice; 30 fibers in both control and Myr-Akt conditions) (Fig. 2*A*). There was increased striatal DA turnover ipsilateral to the side of the AAV Myr-Akt injection, reflected in higher levels of HVA (Fig. 2*B*) and a higher HVA/DA ratio (Fig. 2*B*). Although AAV Myr-Akt-injected mice showed no apparent behavioral motor asymmetries, they did show augmented contraversive rotations after amphetamine injection (Fig. 2*C*), indicating that the sprouting of dopaminergic fibers within the striatum was accompanied by an increased functional capacity to release DA.

Neuroprotective Effects of AAV Myr-Akt in a Neurotoxin Model. In addition to these trophic effects, AAV Myr-Akt was protective in a neurotoxin model of PD. Injection three weeks before intrastriatal 6-hydroxydopamine (6OHDA) conferred near complete protection of DA neuron cell bodies. Although there was

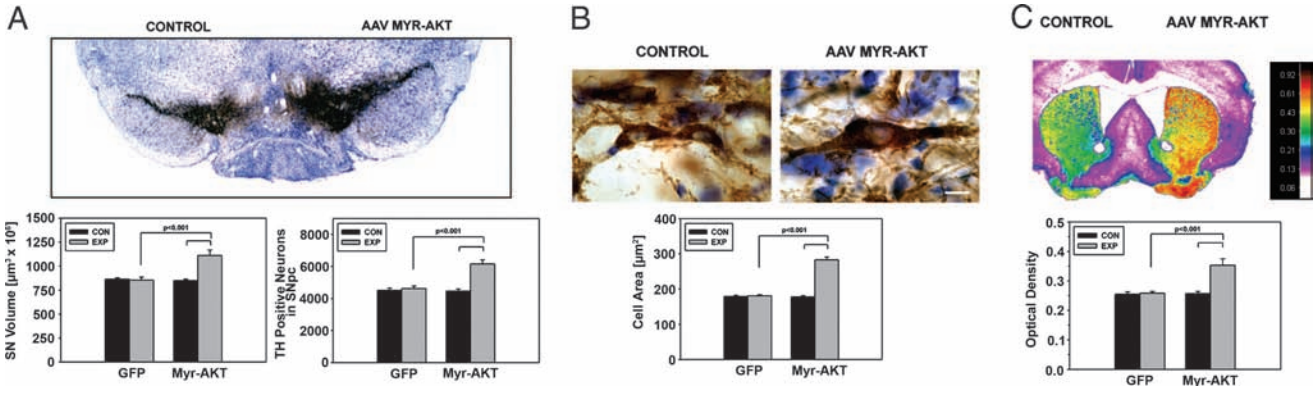

Fig. 4. Trophic effects of Myr-Akt on DA neurons of the SN in aged mice. (*A*) Aged mice were injected with either AAV GFP or AAV Myr-Akt and killed for morphologic analysis 7 weeks later. Myr-Akt induced a 31% increase in the total volume of the SN as compared with the contralateral noninjected SN. There also was an increase in the number of TH-positive neurons; the AAV Myr-Akt-injected SN contained 6,158 ± 253 neurons, a 38% increase above the number of 4,467 ± 131 on the contralateral control side ($P < 0.001$, Tukey's test; $n = 6$ animals). (*B*) As in young mice, Myr-Akt induced an increase in the size of individual TH-positive neurons, as shown. (Scale bar: 10 μm.) (*C*) As in young mice, sprouting was observed with a 37% increase in TH optical density values on the AAV Myr-Akt-injected side in comparison to contralateral control and both striata of AAV GFP-injected mice ($P < 0.001$, Tukey).

a trend for neuron loss on the 6OHDA-injected side (4,036 ± 280 neurons per SN), it was not significantly different from the noninjected control side (4,664 ± 86). This number of neurons represents a more than 4-fold increase over the surviving number in AAV GFP control-injected animals (950 ± 163) (Fig. 3*A*). Increased survival in DA neurons was attributed to a suppression of apoptotic death (Fig. 3*A*).

Myr-Akt also provided protection against axonal loss. At 28 days after 6OHDA, striatal TH optical density levels were reduced only 26% in AAV Myr-Akt-treated mice, whereas they were reduced 74% in AAV GFP-injected controls (Fig. 3*B*) ($P < 0.05$). This morphologic preservation of the dopaminergic striatal projection was accompanied by preservation of biochemical measures of DA and HVA (data not shown). In addition, although the 6OHDA lesion in AAV GFP control mice resulted in an increased rate of DA turnover, indicated by an increased HVA/DA ratio, as previously reported (16), this effect was diminished in the AAV Myr-Akt-injected mice (Fig. 3*C*). This morphologic and biochemical preservation of striatal dopaminergic fibers was functionally significant, because it was associated with diminished apomorphine-induced contraversive rotations, which are attributable to dopaminergic denervation-induced receptor supersensitivity (Fig. 3*D*). The preservation of striatal dopaminergic fibers was a lasting effect, because at 60 days after lesion, density levels were reduced only by 39% in Myr-Akt-treated animals, whereas in GFP controls, they were reduced by 83% ($P < 0.001$) (data not shown).

Although the above experiments demonstrate an ability of Myr-Akt to protect DA neuron cell bodies and axons when given before the neurotoxin, they do not test its effect in circumstances in which dopaminergic dysfunction occurs before therapeutic intervention, as is the clinical situation in PD. To assess Myr-Akt in a paradigm that better simulates the clinical context, we first administered 6OHDA intrastriatally and waited 3 weeks before intranigral injection of AAV Myr-Akt, at which time it is anticipated that ≈40–50% of SN DA neurons remain (Fig. 7, which is published as supporting information on the PNAS web site). In this paradigm, the number of surviving TH-positive neurons at 7 weeks after AAV Myr-Akt injection was 50% of the contralateral control number, whereas the number in AAV GFP-injected controls was reduced to 14% ($P < 0.001$) (Fig. 7). Striatal dopaminergic terminals also were relatively preserved in the Myr-Akt-treated group (Fig. 7).

Trophic Effects of AAV Myr-Akt in Aged Mice. The principal risk factor for PD is increased age (17), and atrophic changes occur in dopaminergic neurons with age (18). We therefore sought to determine whether Myr-Akt has trophic effects on dopaminergic neurons of aged mice. In 22-month-old aged mice, Myr-Akt produced a 31% increase in the volume of the SN (Fig. 4*A*). As in young mice, this effect for the SNpc was attributable, at least in part, to a 60% increase in average neuronal size (Fig. 4*B*). There also was an increase in the number of TH-positive SN dopaminergic neurons (Fig. 4*A*). These neuron size and number effects were not significantly different from those observed in young mice. Aged mice also demonstrated a sprouting response of dopaminergic axons in response to Myr-Akt (Fig. 4*C*), a difference somewhat greater than that observed in young mice but not significantly. We conclude that for most measures, aged mice are as responsive to Myr-Akt as young mice are.

Discussion

These results demonstrate the feasibility of using viral vector transfer to induce trophic responses in normal adult and aged neurons of the central nervous system by direct activation of the intracellular pathways that mediate responses to extracellular neurotrophic molecules. We used an N-terminal Myr form of the survival signaling kinase Akt, which is constitutively targeted to plasma cell membrane and activated (15). This form of Akt had multiple trophic effects on DA neurons of the SN, including (*i*) an increase in neuron size, (*ii*) an increase in the level of expression of TH, and (*iii*) a sprouting response in dopaminergic axons, associated with a functional behavioral correlate.

A number of these effects are in keeping with prior observations on phosphatidylinositide 3′-OH kinase and Akt signaling. Akt signaling previously has been shown to regulate cell size in pancreatic islet cells (19) and cardiac myocytes (20). For neurons, it has been shown that null mutations in PTEN, a negative regulator of phosphatidylinositide 3′-OH kinase-Akt signaling, results in macrocephaly with an increase in neuron soma size (21). Conversely, null mutation of the Akt3 isoform results in smaller brain size attributable to smaller cells (22). However, all of these observations were made in mice with genetic alteration of the germ line, so that the induced mutations were operative throughout development. Our observations are the first to demonstrate the ability of Akt to regulate neuron size in the mature and even aged nervous system.

Akt also has been demonstrated to regulate axonal growth. In culture, Akt expression modifies axon branching of peripheral sensory neurons (13), and, *in vivo*, it regulates the rate of regeneration of peripheral motor nerves after axotomy (14). We

demonstrate here in the central nervous system that Akt also regulates axon sprouting in both adult and aged animals, either in the presence or absence of injury. In both contexts, induced sprouting results in functionally competent nerve endings, because in both normal and 6OHDA-lesioned animals, the Akt-induced increase in dopaminergic striatal innervation had behavioral correlates.

In addition to these trophic effects on normal adult and aged DA neurons, Myr-Akt also demonstrated an ability to block apoptosis in a potent neurotoxin model of parkinsonism, that induced by intrastriatal injection of 6OHDA. We selected this model for several reasons. First, 6OHDA is an endogenous metabolite of DA (23), having been detected in human caudate (24). Thus, to the extent that oxidative metabolism of endogenous DA may play a role in human PD (reviewed in ref. 25), 6OHDA may model this process. Second, among models of PD associated with SN DA neuron death, intrastriatal 6OHDA has been demonstrated unequivocally to induce apoptosis (26). Third, among PD models, the 6OHDA model is the most destructive, typically resulting in over 80% loss of SN DA neurons. And finally, in this model, SN DA neuron loss is progressive (27), like the human disease. The suppression of apoptosis in this model afforded by Myr-Akt was both substantial and lasting, as indicated by an almost complete preservation of the population of SN DA neurons at 28 days after toxin injection. In this respect, Myr-Akt differs from a number of other neuroprotective approaches that suppress cell death in the acute injury period but do not offer increases in DA neuron survival in the long term (28).

To better simulate the clinical setting, we assessed the effects of AAV Myr-Akt-injected at 3 weeks after 6OHDA, by which time ≈50% of DA neurons are lost (27). There was a 50% survival of DA neurons, assessed 10 weeks after 6OHDA (Fig. 7). Although our data are not directly comparable to those of Sauer and Oertel (27), because of species differences, we estimate that in this paradigm AAV Myr-Akt provided substantial protection of remaining viable neurons. In contrast, AAV GFP-injected controls had only 14% neurons surviving at this time point, a near 4-fold reduction.

In addition to its ability to protect DA neuron cell bodies, AAV Myr-Akt also demonstrated an ability to preserve axonal projections, with preservation of both morphologic and biochemical indices of the dopaminergic projection. Function also was preserved as demonstrated by the reduction of apomorphine-induced rotations after 6OHDA. Apomorphine-induced rotations in the 6OHDA model are attributed to the development of postsynaptic DA receptor supersensitivity after disruption of the nigro-striatal projection (29). Therefore, reduction of rotational behavior suggests that supersensitivity has been diminished by partial restoration of striatal DA release in the AAV Myr-Akt-treated animals. This ability of AAV Myr-Akt to preserve the dopaminergic axonal projection distinguishes it as a neuroprotective approach from many others based on blockade of apoptosis that preserves cell bodies but not axonal projections (30).

The relatively sustained expression of Akt1 mRNA, the most abundant isoform in SN, into adulthood, and the marked trophic effects of constitutively active Akt on adult and aged DA neurons suggest the possibility that endogenous Akt may normally play a role in maintaining viability or functionality of these neurons. Such a possibility is of interest in relation to recent observations on DJ-1 (PARK7), in which loss-of-function mutations result in human PD (31). DJ-1 has been shown in *Drosophila* to function as a suppressor of PTEN, a negative regulator of Akt signaling (32). Consistent with these observations, inhibition of DJ-1A in *Drosophila* by RNA interference results in reduced phosphorylation of Akt and degeneration of DA neurons (33). Thus, Akt signaling may be an important molecular mechanism in the pathogenesis of familial PD.

The ability of AAV Myr-Akt to provide neuroprotection in a potent neurotoxin model of PD, particularly its ability to preserve both dopaminergic cell bodies and axonal projections even when administered 3 weeks after the neurotoxin, suggests that it may provide a previously uncharacterized approach to gene therapy in PD. However, Akt is a potent oncogene, and although we did not observe neoplasia in any of the brains examined as long as 81 days after viral injection, it remains possible that chronic expression of a constitutively active form, particularly in nonneuronal brain cells, carries a risk of neoplastic transformation. Nevertheless, with the development of cell-specific expression systems for DA neurons, coupled to the neuronal tropism of AAV, it should be possible to explore the clinical use of Myr-Akt and other factors affecting its function for the treatment of PD.

Methods

Generation of a Recombinant AAV. A plasmid encoding a 5′ src myristoylation signal in frame with mouse Akt1 was kindly provided by Thomas Franke (Columbia University) (34, 35). (Detailed characterization can be found in *Supporting Text*, which is published as supporting information on the PNAS web site.) The Myr-Akt sequence was modified to incorporate a FLAG-encoding sequence at the 3′ end and inserted into an AAV packaging construct as previously described (36). The genomic titer of each virus ranged from 6.1×10^{12} to 1.2×10^{13} viral genomes per ml. Enhanced GFP was subcloned into the same viral backbone, and viral stocks were used at a titer of $4.6–9.1 \times 10^{12}$.

Experimental Animals. Adult (8-week-old) male C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA). Aged male mice (22 months old) were obtained through the National Institute on Aging (Bethesda, MD). For studies of developmental expression of Akt, postnatal rats were used. All injection procedures, described below, were approved by the Columbia University Animal Care and Use Committee.

Virus Injection. Mice were anesthetized with ketamine/xylazine solution and placed in a stereotaxic frame (Kopf Instruments, Tujunga, CA). The tip of 5.0-μl syringe (Agilent, Santa Clara, CA) needle (26S) was inserted to stereotaxic coordinates: AP, −0.35 cm; ML, +0.11 cm; and DV, −0.37 cm, relative to bregma. Viral vector suspension in a volume of 2.0 μl was injected at 0.1 μl/min over 20 min. Characterization of the viral injections is presented in the *Supporting Text*. See Fig. 8, which is published as supporting information on the PNAS web site, for histologically confirmed transduction of DA neurons of the SN with AAV Myr-Akt.

6OHDA Lesion. Adult mice received a unilateral intrastriatal injection of 6OHDA as described in ref. 28 and as detailed in *Supporting Text*.

Immunohistochemistry. Immunostaining for TH was performed as described in ref. 37 and as detailed in *Supporting Text*. Procedures for immunostaining of the FLAG epitope and phosphorylated Akt are described in detail in *Supporting Text*.

Determination of SN DA Neuron Numbers by Stereologic Analysis. Stereologic analysis was performed under blinded conditions on coded slides. For each animal, the SN on both sides of the brain was analyzed. For each section, the entire SN was identified as the region of interest. With StereoInvestigator software (MicroBright Field, Inc., Williston, VT), a fractionator probe was established for each section. The number of TH-positive neurons

in each counting frame then was determined by the optical disector method (38). Further methodologic detail is provided in *Supporting Text*.

Determination of the Number of Apoptotic Profiles Within the SN. Apoptotic profiles were identified in the SN on TH-immunoperoxidase stained and thionin-counterstained sections as previously described (39).

Measurement of SN and Striatal DA and Metabolites. These assays are described in *Supporting Text*.

Western Analysis of TH and the DA Transporter (DAT). These analyses are described in *Supporting Text*.

Behavioral Analysis. Amphetamine-induced rotational behavior in nonlesioned mice was tested at 7 weeks after the viral injection. Mice were injected with amphetamine (5 mg/kg i.p.) (Sigma-Aldrich, St. Louis, MO) and placed free roaming in a plastic hemispherical bowl with a diameter of 26.5 cm. They were allowed to habituate to their environment for 15 min, and then contralateral and ipsilateral turns were counted for 60 min. Results are expressed as net turns per 60 min. Apomorphine-induced rotational behavior was assessed at 4 weeks after lesion. Mice received a s.c. dose of apomorphine (0.5 mg/kg) (Sigma-RBI, Natick, MA), and rotations were monitored by using the same experimental setup as for amphetamine-induced rotation.

Northern and *in Situ* Hybridization Analysis of Akt Isoforms During Development. These analyses are described in *Supporting Text*.

Statistical Methods. Multiple comparisons among groups were performed by one-way analysis of variance and Tukey post hoc analyses.

We thank Ms. Anna Yarygina and Ms. Amy Baohan for morphometric analyses and Dr. Lloyd Greene for his helpful review of the manuscript. This work was supported by National Institutes of Health Grants NS26836, NS38370, and DAMD17-03-1-0492; the Parkinson's Disease Foundation; the Lowenstein Foundation; and the Michael J. Fox Foundation.

1. Mayeux R (2003) *Annu Rev Neurosci* 26:81–104.
2. Lin L-FH, Doherty DH, Lile JD, Bektesh S, Collins F (1993) *Science* 260:1130–1132.
3. Gill SS, Patel NK, Hotton GR, O'Sullivan K, McCarter R, Bunnage M, Brooks DJ, Svendsen CN, Heywood P (2003) *Nat Med* 9:589–595.
4. Lang AE, Gill S, Patel NK, Lozano A, Nutt JG, Penn R, Brooks DJ, Hotton G, Moro E, Heywood P, *et al*. (2006) *Ann Neurol* 59:459–466.
5. Thoenen H, Sendtner M (2002) *Nat Neurosci* 5(Suppl):1046–1050.
6. Davidson BL, Breakefield XO (2003) *Nat Rev Neurosci* 4:353–364.
7. Datta SR, Brunet A, Greenberg ME (1999) *Genes Dev* 13:2905–2927.
8. Kaplan DR, Miller FD (2000) *Curr Opin Neurobiol* 10:381–391.
9. Dudek H, Datta SR, Franke TF, Birnbaum MJ, Yao R, Cooper GM, Segal RA, Kaplan DR, Greenberg ME (1997) *Science* 275:661–665.
10. Soltoff SP, Rabin SL, Cantley LC, Kaplan DR (1992) *J Biol Chem* 267:17472–17477.
11. Creedon DJ, Tansey MG, Baloh RH, Osborne PA, Lampe PA, Fahrner TJ, Heuckeroth RO, Milbrandt J, Johnson EMJ (1997) *Proc Natl Acad Sci USA* 94:7018–7023.
12. Brunet A, Datta SR, Greenberg ME (2001) *Curr Opin Neurobiol* 11:297–305.
13. Markus A, Zhong J, Snider WD (2002) *Neuron* 35:65–76.
14. Namikawa K, Honma M, Abe K, Takeda M, Mansur K, Obata T, Miwa A, Okado H, Kiyama H (2000) *J Neurosci* 20:2875–2886.
15. Pellman D, Garber EA, Cross FR, Hanafusa H (1985) *Nature* 314:374–377.
16. Zigmond MJ, Abercrombie ED, Berger TW, Grace AA, Stricker EM (1990) *Trends Neurosci* 13:290–296.
17. Bower JH, Maraganore DM, McDonnell SK, Rocca WA (2000) *Mov Disord* 15:819–825.
18. Emborg ME, Ma SY, Mufson EJ, Levey AI, Taylor MD, Brown WD, Holden JE, Kordower JH (1998) *J Comp Neurol* 401:253–265.
19. Tuttle RL, Gill NS, Pugh W, Lee JP, Koeberlein B, Furth EE, Polonsky KS, Naji A, Birnbaum MJ (2001) *Nat Med* 7:1133–1137.
20. Shioi T, McMullen JR, Kang PM, Douglas PS, Obata T, Franke TF, Cantley LC, Izumo S (2002) *Mol Cell Biol* 22:2799–2809.
21. Backman SA, Stambolic V, Suzuki A, Haight J, Elia A, Pretorius J, Tsao MS, Shannon P, Bolon B, Ivy GO, *et al*. (2001) *Nat Genet* 29:396–403.
22. Easton RM, Cho H, Roovers K, Shineman DW, Mizrahi M, Forman MS, Lee VM, Szabolcs M, de Jong R, Oltersdorf T, *et al*. (2005) *Mol Cell Biol* 25:1869–1878.
23. Kostrzewa RM, Jacobowitz DM (1974) *Pharmacol Rev* 26:199–288.
24. Curtius HC, Wolfensberger M, Steinmann B, Redweik U, Siegfried J (1974) *J Chromatogr* 99:529–540.
25. Fahn S, Sulzer D (2004) *NeuroRx* 1:139–154.
26. Marti MJ, Saura J, Burke RE, Jackson-Lewis V, Jimenez A, Bonastre M, Tolosa E (2002) *Brain Res* 958:185–191.
27. Sauer H, Oertel WH (1994) *Neuroscience* 59:401–415.
28. Silva RM, Ries V, Oo TF, Yarygina O, Jackson-Lewis V, Ryu EJ, Lu PD, Marciniak SM, Ron D, Przedborski S, *et al*. (2005) *J Neurochem* 95:974–986.
29. Pycock CJ (1980) *Neuroscience* 5:461–514.
30. Eberhardt O, Coelln RV, Kugler S, Lindenau J, Rathke-Hartlieb S, Gerhardt E, Haid S, Isenmann S, Gravel C, Srinivasan A, *et al*. (2000) *J Neurosci* 20:9126–9134.
31. Bonifati V, Rizzu P, van Baren MJ, Schaap O, Breedveld GJ, Krieger E, Dekker MC, Squitieri F, Ibanez P, Joosse M, *et al*. (2003) *Science* 299:256–259.
32. Kim RH, Peters M, Jang Y, Shi W, Pintilie M, Fletcher GC, DeLuca C, Liepa J, Zhou L, Snow B, *et al.* (2005) *Cancer Cell* 7:263–273.
33. Yang Y, Gehrke S, Haque ME, Imai Y, Kosek J, Yang L, Beal MF, Nishimura I, Wakamatsu K, Ito S, *et al.* (2005) *Proc Natl Acad Sci USA* 102:13670–13675.
34. Franke TF, Yang SI, Chan TO, Datta K, Kazlauskas A, Morrison DK, Kaplan DR, Tsichlis PN (1995) *Cell* 81:727–736.
35. Ahmed NN, Grimes HL, Bellacosa A, Chan TO, Tsichlis PN (1997) *Proc Natl Acad Sci USA* 94:3627–3632.
36. Olson VG, Heusner CL, Bland RJ, During MJ, Weinshenker D, Palmiter RD (2006) *Science* 311:1017–1020.
37. Kholodilov N, Yarygina O, Oo TF, Zhang H, Sulzer D, Dauer WT, Burke RE (2004) *J Neurosci* 24:3136–3146.
38. Coggeshall RE, Lekan HA (1996) *J Comp Neurol* 364:6–15.
39. Oo TF, Kholodilov N, Burke RE (2003) *J Neurosci* 23:5141–5148.

Volume 114 No. 3 June 2007 ISSN 0163-7258

Pharmacology & Therapeutics

An International Review Journal

Available online at www.sciencedirect.com
ScienceDirect
Pharmacology & Therapeutics 114 (2007) 261–277
Pharmacology & Therapeutics
www.elsevier.com/locate/pharmthera

Associate editor: M.M. Mouradian

Inhibition of mitogen-activated protein kinase and stimulation of Akt kinase signaling pathways: Two approaches with therapeutic potential in the treatment of neurodegenerative disease

Robert E. Burke *

Departments of Neurology, Pathology, and Anatomy and Cell Biology, Room 306, Black Building, Columbia University Medical Center, 650 West 168th Street, New York, NY 10032, USA

Abstract

The neurodegenerative diseases of adulthood, including Alzheimer's disease (AD) and Parkinson's disease (PD), pose an enormous and growing public health burden. Although effective symptomatic treatments exist for PD, and, to a lesser extent, for AD, there is no therapy for these disorders which will forestall their progression. With the rise of the concept of programmed cell death (PCD) came the realization that even in the absence of complete knowledge of proximate causes neuroprotection may nevertheless be possible by targeting the pathways of PCD. One set of signaling pathways that have been implicated in cell death are the mitogen-activated protein kinase (MAPK) pathways. The possibility of blocking these pathways and thereby providing neuroprotection has recently been put to the test in a clinical trial of a mixed lineage kinase inhibitor in the treatment of PD. Unfortunately, this trial failed to demonstrate a protective effect. Based on considerations related to the implementation of the trial, it would be premature to conclude that inhibition of MAPK signaling is a failed strategy. In spite of these negative results, the MAPK and related kinase pathways retain their importance as potential targets in PD. In relation to pathogenesis, the discovery of mutations in the mixed lineage kinase (MLK)-like kinase leucine-rich repeat kinase 2 (LRRK2) suggests a role for these kinases in regulating the viability of dopamine neurons. In relation to treatment, the survival signaling kinase Akt has been demonstrated in vivo to mediate striking neurotrophic and antiapoptotic effects. Thus, it is likely that therapeutic targets related to these kinase signaling pathways will emerge.

Keywords: Alzheimer's disease; Parkinson's disease; c-jun; JNK; Mixed lineage kinases; Akt; LRRK2

Abbreviations: 6OHDA, 6-hydroxydopamine; AAV, adeno-associated virus; AD, Alzheimer's disease; AGC kinases, PKA, PKG, AND PKC related kinases; AP-1, activator protein-1; ASK1, apoptosis signaling kinase-1; DLK, dual-leucine-zipper-bearing kinases; JBD, JNK binding domain; JIP1, JNK interacting protein-1; JNK, c-jun N-terminal kinase; LRRK2, leucine-rich repeat kinase 2; MAPK, mitogen-activated protein kinase; MLK, mixed lineage kinase; mTOR, mammalian target of rapamycin; NGF, nerve growth factor; PCD, programmed cell death; PD, Parkinson's disease; PDGF, platelet-derived growth factor; PDK1, 3-phosphoinsitide-dependent kinase 1; PH, pleckstrin homology domain; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; POSH, plenty of SH3; PTEN, phosphatase and tensin homologue deleted on chromosome ten; SCG, superior cervical ganglion; SN, substantia nigra; TH, tyrosine hydroxylase; TNF, tumor necrosis factor; TSC1/2, tuberous sclerosis complex 1 and 2.

Contents

* Tel.: 212 305 7374; fax: 212 305 5450.
E-mail address: rb43@columbia.edu.

0163-7258/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.pharmthera.2007.02.002

1. Introduction

1.1. Epidemiology and current treatment of neurodegenerative diseases

Neurodegenerative diseases of adulthood, including Alzheimer's, Parkinson's, and motor neuron disease as 3 principal examples, represent an enormous and rapidly growing public health burden globally. These disorders are estimated to affect 20 million individuals worldwide (Mayeux, 2003). Each of these diseases increases in its incidence with age, and so with the steady increase in the average age of populations, particularly in developed nations, the morbidity and economic burdens which they impose will grow. Alzheimer's disease (AD), which manifests principally as a steady decline in cognitive abilities, increases virtually exponentially in its prevalence with age. Less than 1% of individuals aged 60–64 years are affected, whereas 24–33% of individuals 85 years or older are (Blennow et al., 2006). Medical costs for patients with AD are predicted to increase from $91 billion in 2005 to $160 billion by 2010 (Alzheimer's Association Fact Sheet). Parkinson's disease (PD) manifests initially mainly with impairments of motor function, including tremor, rigidity, slowness of movement and postural instability, but with time, these patients develop nonmotor impairments, including dementia, depression and autonomic failure. Approximately 1 million Americans are estimated to be affected with PD (Mayeux, 2003). Like AD, its incidence increases exponentially with age (Bower et al., 2000). These figures do not reflect, of course, the emotional and personal devastation which these diseases visit upon patients and their families. Nor do they reflect the health burdens imposed by common, but often unmentioned, related disorders; the cognitive decline caused by the frontotemporal dementias or diffuse Lewy body disease, or the Parkinson-like impairments caused by progressive supranuclear palsy, the multiple system atrophies, corticobasal ganglionic degeneration, and many others.

In view of the increasing magnitude of these problems, there is desperate need to develop therapies which will halt or hopefully even reverse their inexorable progression. At present there are none for any of them. On the basis of experimental evidence that cognitive impairment in AD is due, at least in part, to the degeneration of cholinergic neurons of the basal forebrain, pharmacologic approaches have been directed towards enhancement of neurotransmission by acetylcholine. A number of anticholinesterases that are effective in providing some cognitive improvement in mild to moderate AD are now available, but they, of course, do not modify the progression of the disease (Blennow et al., 2006).

There are many effective medical and surgical therapies for the symptomatic treatment of the motor signs of PD (reviewed in Lang & Lozano, 1998), but they often lose efficacy and develop complications in the long term. They do not benefit, and in some instances exacerbate, disabling nonmotor features of the disease. As is the case for AD, there is no therapy currently available, which prevents the inexorable worsening of the disease due to progressive neurodegeneration.

For both AD and PD, great strides have been made in recent years in our understanding of the molecular basis of neurodegeneration, and these advances have illuminated many possible molecular targets for the development of neuroprotective approaches. For both disorders, the clearest advances have been made possible by the discovery of the genetic causes of the relatively less common familial cases. While the familial forms of these diseases may be less common than the sporadic forms, the molecular insights into possible disease mechanisms provided by the identification of specific disease-causing mutations provide powerful tools. In PD, for example, in the past 10 years disease-causing mutations have been identified in 6 different genes (reviewed in Cookson, 2005; Moore et al., 2005; Jain et al., 2005). Importantly, many of these discoveries have suggested common themes in pathogenesis, including protein aggregation and the mishandling of cellular proteins and abnormalities in mitochondrial function. These important advances notwithstanding, it is important in the experimental development of neuroprotective strategies to recognize that what we clinically diagnose as PD may have diverse proximate causes, especially among the sporadic forms, so neuroprotective approaches based on select "upstream" mechanisms of disease may not benefit all patients. Furthermore, we may find that, in spite of our best efforts, strategies directed at proximate causes may not yield to attempts at therapeutic intervention. Therefore, in the development of neuroprotective approaches, it is worthwhile to target molecular mechanisms of neuron death which appear to be shared by diverse forms of neurologic disease: the pathways of programmed cell death (PCD).

1.2. Programmed cell death

The concept that the molecular pathways of PCD may participate in the processes of chronic neurodegeneration emerged about 15 years ago (Oppenheim, 1991; Ellis et al., 1991; Johnson & Deckwerth, 1993; Thompson, 1995) and this hypothesis remains under consideration for many of the major neurodegenerative diseases including AD and PD. Although the precise role of any specific pathway of PCD remains to be known for these diseases, the concept of PCD has provided a new way of thinking about neuroprotection for them. Even in the absence of exact knowledge of the proximate causes of neurodegeneration, and even in the presence of diverse possible causes, it may be feasible to abrogate neuron death by inhibition of these widely shared pathways.

Several theoretical concerns are often raised about the efficacy of targeting the pathways of PCD. There has been concern that they may be too far "downstream" to preserve sufficient cellular functionality if they are blocked. There has also been concern that these pathways are so redundant and diverse that any attempt, either pharmacologic or genetic, to block them will be futile. And finally, there is the consideration that blocking PCD may protect the neuron cell body, but do little to protect axons, which are likely to degenerate due to molecular mechanisms distinct from those which play a role in PCD (Raff et al., 2002). While all of these concerns are valid, they must be put to empirical test, and there is no a priori reason to believe that they are insurmountable.

Among the diverse and numerous pathways of PCD (reviewed in Bredesen et al., 2006), there is now an abundance of evidence that the c-jun N-terminal kinase (JNK) signaling cascade may be a therapeutic target in neuron death, including that of dopamine neurons, one of the populations of neurons which degenerate in PD. This preclinical evidence has led to the first trial of a drug which blocks PCD to attempt to provide neuroprotection in a neurodegenerative disease; the PRECEPT trial of the mixed linage kinase (MLK) inhibitor CEP1347 in the treatment of PD. In spite of preclinical data reviewed and summarized in Sections 2.2 and 2.3 below, this trial has unfortunately failed to find benefit, and it has brought this strategy to a crossroad (Waldmeier et al., 2006). We will comment on the trial (unpublished at the time of this review) and consider its implications for future approaches based on inhibition of the JNK signaling cascade.

Independent of this theme relating the JNK cascade to regulation of the viability of dopamine neurons, an additional theme has emerged to suggest the importance of kinase signaling in the control of the viability of dopamine neurons, and possibly other neuronal populations as well. We have recently found that the survival signaling kinase Akt/protein kinase B (PKB) has striking trophic effects in vivo on dopamine neuron viability and functionality. These results demonstrate that the many trophic effects of Akt observed in vitro generalize to the in vivo context in a neuronal population selectively vulnerable to PD. We will review herein the mechanisms of Akt signaling and how they may provide future neuroprotective approaches to PD.

2. The role of c-jun N-terminal kinase signaling in neuron death

2.1. Overview of the mixed lineage kinase-c-jun N-terminal kinase signaling pathways

JNK (also known as stress-activated protein kinase, or SAPK) is a member of a family of kinases called the mitogen-activated protein kinases (MAPK) which also includes the p38 and the extracellular signal-regulated kinases (ERK; Davis, 1994; Kyriakis & Avruch, 2001). All eukaryotic cells possess multiple MAPK pathways which are activated by a wide range of stimuli including hormones and growth factors, inflammatory cytokines, and diverse environmental stresses (Kyriakis & Avruch, 2001). These pathways, in turn, have a variety of downstream effects, including the regulation of gene transcription, the cell cycle, cellular differentiation and cell death. All MAPK signaling pathways are organized in a 3-tier signaling structure (Fig. 1) in which the MAPK (in the first tier) are activated by phosphorylation of Tyr and Thr residues within a conserved Thr-X-Tyr motif. This phosphorylation is catalyzed by upstream kinases (the second tier), the MAPK kinases (MAPKK), also known as the MAPK/ERK kinases (MEK or MKK; Davis, 2000; Kyriakis & Avruch, 2001). These kinases are, in turn, regulated by Ser/Thr phosphorylation, which is catalyzed by any of several MAPK kinase kinase (MAPKKK) families. Regulation of MAPKKK is achieved by membrane recruitment, oligomerization and phosphorylation. In some cellular contexts, this is mediated by GTPases of the Ras

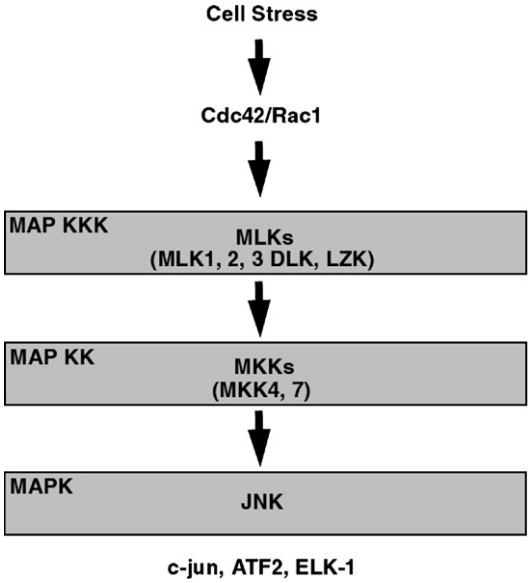

Fig. 1. The MAPK signaling pathways. MAPK signaling pathways are organized in a 3-tier structure. JNK, which mediates phosphorylation and activation of c-jun, is a MAPK in the first tier. Also in this group of kinases are the p38 MAPK and the ERKs (not shown). The MAPK are activated by phosphorylation of Tyr and Thr residues by the MAPK kinases in the second tier. In this tier, MKK4 and MKK7, in particular, mediate activation of JNK. Upstream to the MAPKK, in the third tier, several families of kinases have been reported to activate JNK (see the text). Among these kinases, the MLK have been implicated in neuron death.

superfamily, such as Cdc42 and Rac proteins. A fundamental organizational principal of the MAPK pathways is that their complexity is managed in part by scaffold proteins which are capable of binding, sequestering, and fostering specific interactions among selected components. This organization permits specific types of stimuli to produce unique and coordinated MAPK signaling responses (Kyriakis & Avruch, 2001).

JNK was first identified in 1990 as a rat hepatic kinase for microtubule-associated protein 2 (Kyriakis & Avruch, 1990). It was cloned in 1994 (Kyriakis et al., 1994; Derijard et al., 1994). There are 3 genes encoding JNK protein kinases. The *Jnk1* and *Jnk2* genes are widely expressed, whereas *Jnk3* is expressed only in brain, heart and testis (Davis, 2000). Alternate splicing of the gene transcripts results in further molecular diversity. A splice site within the Jnk1 and 2 transcripts results in 2 splice forms; a second alternate splice for all JNK transcripts occurs at the C terminus of the protein resulting in the 46 and 55 kDa protein isoforms. Thus, 10 isoforms have been identified. JNK has numerous substrates, but it is the dominant kinase for c-jun *in vivo*; immunodepletion of JNK from cell extracts removes all stress and cytokine-activated c-jun phosphorylation activity (Kyriakis & Avruch, 2001).

Upstream activation of the JNK is mediated primarily by 2 MAPKK, MKK4 (also know as SAPK/ERK kinase-1 or SEK1) and MKK7. Gene disruption of MKK4 and 7 eliminates JNK activation, indicating that they are major activators of JNK in vivo (Tournier et al., 2001). These 2 kinases act synergistically to phosphorylate and activate the JNK. In vitro, each kinase alone activates JNK about 5- to 10-fold, but when added together, they activate them about 100-fold (Kyriakis & Avruch, 2001). In addition, they appear to mediate JNK activation by different stimuli; MKK7 is activated primarily by cytokines [such as tumor necrosis factor (TNF) and IL-1], whereas MKK4 is primarily activated by environmental stress (Davis, 2000).

Upstream to the MAPKK, several families of MAPKKK have been reported to activate JNK, including the MEK kinases (MEKK1–4; MEK is an alternate name for the MKK), apoptosis signaling kinase-1 (ASK1), transforming-growth factor ß-activated kinase1 (TAK1), tumor progression locus-2 (TPL-2), and MLK (see Davis, 2000; Kyriakis & Avruch, 2001 for complete reviews). The MLK have been clearly implicated in PCD in neurons based on studies with the MLK inhibitor CEP1347 (Maroney et al., 2001) and dominant negative genetic approaches (Xu et al., 2001), so we will limit this review to this family of JNK activators.

In general, protein kinases contain 11 conserved subdomains. Of these, subdomains I–VII of the MLK resemble serine/threonine kinases, whereas subdomains VIII–XI more closely resemble tyrosine kinases. Thus, when the MLK genes were initially cloned, they were termed "mixed lineage kinases." However, biochemical studies have shown that the MLK serve as serine/threonine kinases. In recent years, 3 subfamilies of MLK have been identified containing 7 different kinases (Gallo & Johnson, 2002). Based on an analysis of domain arrangements and sequence similarities, the 3 families are the MLK (containing MLK 1-4), the dual-leucine-zipper-bearing kinases (DLK; containing DLK and leucine-zipper kinases), and the zipper

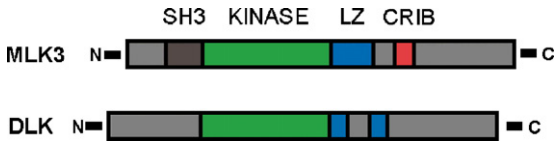

Fig. 2. The protein domain structures of MLK3 and DLK. MLK3 and DLK provide representative examples of the domain structures of the MLK. Common to all MLK is the kinase domain (green), which mediates phosphorylation, and the leucine zipper domain (blue), which mediates protein-protein dimerization. Exemplified here by MLK3, the MLK1–4 contain an N-terminal SH3 domain (brown), which may be involved in autoregulatory mechanisms. MLK1–4 also contain a CRIB motif (red) that is believed to mediate activation by Cdc42.

sterile-α-motif kinases (ZAK; containing ZAK alone). In rat substantia nigra (SN), mRNA for DLK is far more abundant than that for MLK1–3 or LZK (Ganguly et al., 2004), but in human SNpc MLK3 is the most abundant (Kholodilov et al., 2006). The domain structure for the MLK families can be exemplified by that of MLK3 and DLK (Fig. 2). MLK1–4 have an amino terminal Src-homology-3 (SH3) domain, which apparently serves to autoinhibit the kinase activity (Gallo & Johnson, 2002). Moving towards the C terminal, this domain is followed by the kinase domain, containing the catalytic site for phosphorylation. There then follows a leucine zipper region, important for protein-protein interactions. It has been shown for DLK that the leucine zipper is required for self-association, phosphorylation, activation and stimulation of the JNK pathway (Gallo & Johnson, 2002). An MLK3 mutant lacking the zipper fails to autophosphorylate and activate JNK (Gallo & Johnson, 2002). In MLK1–4, following the leucine zipper towards the C terminal, there is a Cdc42/Rac-interactive binding motif (CRIB), which mediates interaction with these Rho family GTPases. MLK3, for example, is able to bind to activated forms of Cdc42 and Rac; when Cdc42 and MLK 3 are coexpressed in cells, there is an increase in MLK3 activity and potentiation of JNK activation. All of the known members of the MLK family, when transfected into cells, act as MAPKKK to activate JNK, and they do so by activation of MAPKK such as MKK4 and MKK7 (Kyriakis & Avruch, 2001; Gallo & Johnson, 2002). While the MLK are principally known for their ability to activate the JNK (Kyriakis & Avruch, 2001), some have been shown to also activate p38 (Gallo & Johnson, 2002).

In the context of JNK activation by cytokines, such as members of the TNF family, the MAPKKK are activated by the binding of the cytokine ligand to cell surface receptors of the TNF receptor superfamily (Davis, 2000; Kyriakis & Avruch, 2001). Binding induces oligomerization with signaling proteins, the TNFR-associated factor (TRAF) proteins either directly, as in the case of TNFR2, or indirectly through a TNFR-associated death domain protein (TRADD), as is the case for TNFR1. The TRAF, in turn, can activate JNK via MAPKKK (Kyriakis & Avruch, 2001). One of the TRAF, TRAF2, has also been shown to mediate the activation of JNK in the setting of endoplasmic reticulum stress (Urano et al., 2000).

The ability of JNK to phosphorylate c-jun and thereby enhance its ability to transactivate other genes (Hibi et al., 1993) remains its principal recognized role (Davis, 2000). C-jun dimerizes with itself and other transcription factors (such as c-*fos*

and ATF) to constitute activator protein-1 (AP-1) transcription factors which regulate the expression of a number of stress-responsive genes. N-terminal phosphorylation of c-jun increases its stability (Musti et al., 1997). JNK also phosphorylates and activates other AP-1 proteins, including JunB, JunD and ATF2. In addition, another transcription factor, ElK1, is a target of JNK phosphorylation; it is involved in induction of the c-*fos* gene. Thus, the role of JNK in mediating PCD is likely to be, at least in part, through its role in regulating gene transcription, and this role is likely to be mediated primarily through its ability to phosphorylate c-jun. This conclusion is supported by the observation that mutations of c-jun, substituting alanines for its serine 63 and 73 phosphorylation sites, lead to increased resistance to apoptosis in neurons (Behrens et al., 1999), as discussed further below.

However, JNK mediates its effects on apoptosis not only through its effects on gene transcription. There is increasing evidence that JNK is able to directly phosphorylate and regulate pro- and antiapoptotic activity of members of the Bcl-2 family. A number of studies have demonstrated that JNK can phosphorylate and diminish the antiapoptotic activity of both Bcl-2 (Maundrell et al., 1997; Yamamoto et al., 1999) and Bcl-X_L (Kharbanda et al., 2000). The latter study demonstrated that induction of apoptosis by irradiation is associated with translocation of JNK to mitochondria and binding to Bcl-X_L. In addition, JNK is capable of phosphorylating the proapoptotic protein BAD at serine 128 and potentiating its proapoptotic effect (Donovan et al., 2002). Similarly, JNK has been demonstrated to phosphorylate the proapoptotic proteins Bim and Bmf, thereby causing their release from sequestration by dynein motor complexes, with translocation to mitochondria, followed by release of mitochondrial death mediators (Lei & Davis, 2003).

A general principle in the organization of the MAPK signaling cascades is that protein interactions are orchestrated in part by scaffolding proteins which are able to bind specific protein components and foster interactions among them, thus permitting specific stimuli to produce unique signaling responses. This principle can be illustrated for JNK signaling by 2 such scaffolding proteins: JNK interacting protein-1 (JIP1; Dickens et al., 1997) and plenty of SH3 (POSH; Tapon et al., 1998).

JIP1 was first identified as a specific JIP; it does not interact with the p38s or the ERK (Dickens et al., 1997). As predicted for a scaffolding protein, it is able to bind multiple components involved in JNK activation: among the MAPKKK, it interacts with MLK3 and DLK; among the MAPKK, it interacts with MKK7. Coexpression of JIP1 with MLK3 or MKK7 enhances the ability of these kinases to activate JNK. JIP1 illustrates the role of scaffolding proteins to regulate the specificity of MAPK signaling, because JIP1 null animals show deficits in the activation of JNK due to excitotoxic stress or anoxia, but their neurons do not show deficits in activation due to UV radiation (Whitmarsh et al., 2001). Expression of the JNK binding domain (JBD) alone (aa 127–281) has dominant negative effects (Dickens et al., 1997), and it has been used *in vivo* to demonstrate the role of JNK signaling in MPTP toxicity (see below; Xia et al., 2001).

POSH was first identified as a Rac-interacting protein by the yeast 2-hybrid method, and the interaction was shown to be GTP dependent (Tapon et al., 1998). POSH was shown to induce JNK activation and apoptosis in nonneuronal cells (Tapon et al., 1998). Xu et al. (2003) demonstrated that POSH expression induces JNK phosphorylation and apoptosis in neural cells. Furthermore, it is capable of direct interaction with the MLK, MKK4 and 7 and the JNK, and, as would be predicted, it acts upstream to the MLK, the MKK and c-jun to induce cell death (Xu et al., 2003).

2.2. Mitogen-activated protein kinase signaling in neuronal cell death in vitro

Over the last decade, a large body of in vitro evidence has emerged suggesting that c-jun plays an important role in the mediation of neuronal PCD. One of the principal in vitro models for the study of cell death in neurons has been the use of sympathetic neurons isolated from rat superior cervical ganglia (SCG). Following the withdrawal of nerve growth factor (NGF), these neurons undergo apoptosis (Martin et al., 1988; Deckwerth & Johnson, 1993; Edwards & Tolkovsky, 1994). Early studies demonstrated upregulation of c-jun protein (Ham et al., 1995) and mRNA (Estus et al., 1994) in this model. Microinjection of a dominant-negative form of c-jun protected SCG neurons from NGF withdrawal-induced cell death, whereas overexpression of wild-type c-jun protein resulted in significant induction of apoptosis even in the presence of NGF (Ham et al., 1995). Similarly, microinjection of neutralizing antibodies for c-jun protein significantly reduced neuronal death following NGF withdrawal (Estus et al., 1994). Identical treatment using neutralizing antibodies directed against other members of the AP-1 family, such as JunB and JunD, failed to demonstrate a protective effect. These studies clearly illustrated that c-jun plays a significant role in the death of sympathetic neurons following trophic factor withdrawal. Similar results have also been reported in cerebellar granule cells following survival signal withdrawal (Watson et al., 1998), and in differentiated PC-12 cells following NGF withdrawal (Mesner et al., 1992).

Activation of the c-jun pathway upon induction of PCD has also been identified in tissue culture in cells that more closely resemble the dopaminergic phenotype. Holtz and O'Malley (2003) have investigated MN9D cells, which display many properties of living dopaminergic neurons including the synthesis and storage of dopamine (Choi et al., 1991) and primary mesencephalic cultures. They identified upregulation of both c-jun mRNA and c-jun phosphorylation in MN9D cells and increased phosphorylation in primary cultures, following treatment with the catecholamine-specific neurotoxin 6-hydroxydopamine (6OHDA).

As the principal kinase for c-jun, JNK has also been implicated in PCD in tissue culture models. Withdrawal of NGF from PC12 cells leads to sustained activation of JNK (Xia et al., 1995). In SCG cells, withdrawal of NGF induces increased JNK activity, serine 63 phosphorylation of c-jun, and transcriptional activation of the c-jun promoter (Eilers et al., 1998). Prevention

of c-jun phosphorylation protects PC-12 cells from apoptosis following NGF withdrawal (Le Niculescu et al., 1999).

Upstream regulators of JNK activity, such as members of the MLK family have also been shown to modulate c-jun activation and apoptosis. Overexpression of MLK induced apoptotic cell death in PC12 cells (Xu et al., 2001) and SCG neurons (Mota et al., 2001). Expression of dominant negative MLK blocked NGF withdrawal-induced apoptosis (Xu et al., 2001; Mota et al., 2001).

The possibility of targeting the MLK in particular for neuroprotective therapeutics has been supported by studies with the MLK inhibitor CEP1347. CEP1347 is a derivative of the indolocarbazole alkaloid K-252a, a natural product isolated from *Nocardiopsis* bacteria, which had previously been shown to promote cell survival in vitro (Knusel & Hefti, 1992). CEP1347 has been shown to be neuroprotective in vitro using an array of cellular insults (reviewed in Saporito et al., 2002). Maroney et al. (1998) demonstrated that, following trophic factor withdrawal, rat motoneuron cultures treated with CEP1347 displayed enhanced survival. Survival in this study was shown to correlate with the inhibition of JNK1 activity. These findings were confirmed and extended in a subsequent study demonstrating that pretreatment with CEP1347 prevented NGF withdrawal-, UV irradiation- and oxidative stress-induced death in neuronally differentiated PC-12 cells and rat sympathetic neurons (Maroney et al., 1999). The fact that these cellular insults induce death via 3 distinct pathways suggested that the mechanism of CEP1347 involved a shared molecular component, most likely, the activation of the JNK pathway (Saporito et al., 2002).

The role of the JNK cascade in CEP1347-mediated neuroprotection was further elucidated by findings demonstrating that CEP1347 inhibited both JNK activation and cell death induced by members of the MLK family in vitro (Maroney et al., 2001). These findings suggested that the protective effect of CEP1347 was a result of the inhibition of the MLK. Mathiasen et al. (2004) expanded these findings by demonstrating that treatment with CEP1347 or a dominant-negative MLK3 adenoviral construct inhibited MPP+-induced cell death as well as JNK signaling in neuronally differentiated human neuroblastoma SH-SY5Y cells.

2.3. MAPK signaling in neuronal cell death in vivo

Initial studies of c-jun expression in the central nervous system of living animals in models of injury were difficult to interpret in relation to cell death, because early studies in peripheral systems had shown that expression could be upregulated by regenerative processes (Jenkins & Hunt, 1991). Such was also the case in some contexts of central injury; in a fimbria-fornix axotomy model, for example, in which death of medial basal forebrain neurons does not occur, there is a sustained increase in c-jun mRNA and protein expression (Haas et al., 1996). Therefore, in the earliest studies of c-jun expression at the regional level in injury models accompanied by neuron death, it was difficult in the diverse neuronal populations to specifically attribute cell death to c-jun expression. Nevertheless, early studies in a variety of ischemia models noted close associations in time and regional location between c-jun mRNA or protein expression and neuron death (Wessel et al., 1991; Dragunow et al., 1993; Gubits et al., 1993). One particular study, by Dragunow and co-workers, noted expression of c-jun in neurons undergoing a delayed neuronal death as opposed to early necrotic death in a neonatal hypoxia-ischemia model, and suggested that the former may be a form of PCD (Dragunow et al., 1994).

The earliest studies specifically within the SN in models of death induced by 6OHDA (Jenkins et al., 1993) and by axotomy (Leah et al., 1993) noted substantial and sustained increases in c-jun expression but these changes were interpreted to be related to a role in regeneration. In the 6OHDA model, however, the maximal expression of c-jun, at 4-8 days post-lesion (Jenkins et al., 1993) is when other investigators subsequently showed that cell death is maximal (Sauer & Oertel, 1994).

With increased awareness of apoptosis as a distinct morphology of PCD (Kerr et al., 1995), and the ability to detect it by nuclear staining, it became clear that c-jun expression could be correlated at the cellular level with this form of cell death in living animals. This was true in the context of naturally occurring cell death in the peripheral (Messina et al., 1996) and central (Ferrer et al., 1996b) nervous systems, and in models of induced naturally occurring cell death (Ferrer et al., 1996a). Similarly, in the SN, close correlations could be made between c-jun expression and markers of apoptosis. Herdegen et al. (Herdegen et al., 1998) demonstrated in the adult axotomy model a close regional and temporal association between prolonged c-jun expression and TUNEL labeling for apoptosis (Gavrieli et al., 1992). Oo et al. (Oo et al., 1999) demonstrated in a postnatal model of apoptosis in the SNpc, induced by early target deprivation, that c-jun and JNK expression could be correlated at a cellular level with apoptotic morphology. Thus, these morphologic studies of apoptotic cell death suggested a clear correlation with c-jun expression.

The first principal evidence for a functional role for JNK/c-jun signaling in cell death in living animals derived from studies in JNK null animals. Yang and co-investigators reasoned that since the JNK3 isoform is selectively expressed in the nervous system, it may play a role in neuronal death. They showed that JNK3 null mice are indeed resistant to kainic acid-induced seizures and associated hippocampal neuron apoptosis (Yang et al., 1997). These animals also demonstrated diminished levels of c-jun phosphorylation and AP-1 transcriptional activity. While this study demonstrated a clear role for this JNK isoform in mediating cell death, it remained an open question whether c-jun itself was the relevant substrate for this effect. To address the precise role of c-jun, Behrens and colleagues created mice by homologous recombination in which the endogenous c-jun gene was replaced by an altered gene in which the serines at positions 63 and 73 were replaced by alanines, which cannot be phosphorylated (Behrens et al., 1999). Mice homozygous for this mutant, non-phosphorylatable form of c-jun were also resistant to seizures and hippocampal neuron apoptosis induced by kainate. Thus, the phosphorylation of c-jun by JNK appears to be responsible for apoptosis in this model.

A functional role for c-jun in mediating death specifically within dopamine neurons has been supported by studies using viral vector gene transfer approaches. Crocker et al. (Crocker et al., 2001) have demonstrated in an axotomy model that adenovirus-mediated expression of a c-jun dominant-negative construct not only prevents the loss of dopamine neurons in the SN, but also the loss of dopaminergic fibers in the striatum. A functional role for JNK/c-jun signaling in dopamine neuron death is also supported by the demonstration that gene transfer of the JBD of JIP-1 (which inhibits JNK activation) protects dopamine neurons from chronic MPTP toxicity (Xia et al., 2001). Again, this approach not only prevented the loss of SN dopamine neurons, but also their striatal terminals, as assessed by catecholamine levels.

In view of this evidence that phosphorylation of c-jun plays a role in the mediation of cell death in dopamine neurons, it would be anticipated that JNK isoforms would also play a role. Hunot and co-investigators have shown in a model of acute MPTP toxicity that both JNK2 and JNK3 homozygous null animals are resistant; each genotype shows only about a 50% reduction of SN dopaminergic neurons, much less than controls (Hunot et al., 2004). JNK1 null animals were not protected. Compound mutant JNK2 and 3 homozygous nulls were even more protected showing only a 15% loss of neurons. Thus both JNK2 and JNK3 play a role in cell death in this model. The compound null mutation also protected dopaminergic fibers in the striatum. These investigators postulated that increased transcriptional activity mediated by JNK phosphorylation of c-jun may mediate cell death, and they found, by microarray analysis, that the immune mediator cyclooxgenase-2 is upregulated. JNK was shown to be necessary for this upregulation, as it was abolished in the compound JNK mutants. Thus, JNK may ultimately act, at least in part, in the acute MPTP model by upregulation of cycloogenase-2, which has been implicated as a death mediator in this model (Teismann et al., 2003).

These results cannot, however, be construed as direct evidence of a role for JNK as a cell-autonomous mediator of apoptotic death within dopamine neurons. The principal reason is that apoptosis does not occur in the acute MPTP model (Jackson-Lewis et al., 1995), whereas it does in the chronic MPTP model (Tatton & Kish, 1997). Another important difference between the 2 models is that a major inflammatory component occurs in the acute model, whereas it is much less in the chronic model (Furuya et al., 2004). Therefore, while it is clear that JNK plays an important role in dopamine neuron death in the presence of inflammation in the acute model, it remains to be determined if it is necessary for cell-autonomous apoptotic death within dopamine neurons.

These studies, based on either gene transfer or transgenesis in mice, indicating a functional role for MAPK signaling in the mediation of neuron death in living animals, have received much support from pharmacologic studies using the specific MLK inhibitors CEP1347, described earlier, and its analogue CEP11004 (Murakata et al., 2002). As summarized above, there is much evidence that CEP1347 can abrogate PCD in a variety of tissue culture models utilizing many different types of cellular insult. Efficacy of CEP1347 to forestall PCD has also been observed in diverse living animal models. Glicksman et al. demonstrated that application of CEP1347 prevented naturally occurring cell death in spinal motor neurons in both embryonic chicks and postnatal rats (Glicksman et al., 1998). This compound has also been demonstrated to forestall pathologic cell death in injury models. In an excitotoxic injury model, induced by intracerebral injection of ibotenate, CEP1347 protected basal forebrain cholinergic neurons (Saporito et al., 1998). In a model of apoptosis induced in auditory hair cells by noise trauma, CEP1347 diminished the loss of cells, and protected hearing (Pirvola et al., 2000).

These MLK inhibitors have also been shown to be protective in animal models of parkinsonism. In a single dose model of MPTP toxicity, Saporito and co-investigators demonstrated that CEP1347 attenuated the loss of dopaminergic terminal markers and cell bodies in SN (Saporito et al., 1999). In the MPTP single dose model, there is increased phosphorylation of JNK and the upstream kinase MKK4, and these increases are attenuated by CEP1347 (Saporito et al., 2000). A similar ability to inhibit the phosphorylation of MKK4 and prevent the loss of dopaminergic terminals in the single dose MPTP model was also demonstrated for CEP11004 (Murakata et al., 2002). In the acute MPTP model, Teismann et al. (Teismann et al., 2003) demonstrated that CEP11004 inhibited phosphorylation of c-jun, diminished the loss of tyrosine hydroxylase (TH)-positive neurons, and prevented increases in cyclooxygenase-2. Since these models did not directly examine the occurrence of cell death, and since, as discussed above, apoptosis does not occur in the acute MPTP model, it remained to be determined whether MLK inhibition could directly forestall apoptotic death within SN dopamine neurons. In a model characterized by the exclusive induction of apoptosis in these neurons by intrastriatal injection of 6OHDA in postnatal rats, CEP11004 diminished the number of dopaminergic apoptotic profiles (Ganguly et al., 2004). This death is mediated, at least in part, by the intrinsic mitochondrial pathway, because it is associated with an induction of the activated cleaved form of caspase 9. CEP11004 acts upstream to this point, because it diminishes the number of caspase-9-positive profiles in proportion to overall protection from cell death (Ganguly et al., 2004). A notable result of this study was an almost complete protection of striatal TH-positive fibers.

The ability of CEP1347 to protect SN dopamine neurons from MPTP has also been demonstrated in primates (Saporito et al., 2002). Cynomologous monkeys were administered MPTP on a weekly basis, and those treated with CEP1347 showed less parkinsonism than vehicle-treated animals. In addition, they showed a preserved number of SN dopamine neurons. Overall, these studies demonstrate a clear neuroprotective effect of these MLK inhibitors in a variety of living animal models of parkinsonism.

2.4. Mitogen-activated protein kinase signaling in human postmortem brain in neurodegenerative disease

To date, evidence for the involvement of c-jun in human neurodegenerative disease has been limited. Nevertheless, a number of studies have implicated this pathway in the

pathophysiology of human neurodegenerative disease, particularly AD. Anderson et al. first reported increased intensity of c-jun immunostaining in the hippocampus and entorhinal cortex of AD brains, in comparison to controls (Anderson et al., 1994). The c-jun immunoreactivity was colocalized with staining for paired helical filaments. These investigators subsequently also observed a relationship between c-jun immunostaining and TUNEL labeling for DNA strand breaks (Anderson et al., 1996). MacGibbon et al. (1997) also found some evidence for increased immunostaining for c-jun in AD postmortem hippocampus. Using a quantitative approach, Marcus et al. (1998) also identified an increased number of c-jun-positive profiles in AD hippocampus, as compared to age-matched controls. Such differences were not observed in a control region, the cerebellum.

Evidence of a role for MAPK signaling in PD brain has been mixed. Recently, Hunot et al. (2004) have reported evidence of c-jun activation in postmortem tissue from idiopathic PD patients. A quantitative analysis of c-jun-positive, pigmented neurons in SNpc revealed that a greater proportion showed a nuclear localization among the PD patients. Ferrer et al. (2001) identified phosphorylated JNK immunoreactivity rarely in the cytoplasm of some neurons, in the vicinity of Lewy bodies, in the brain stem of patients with PD or dementia with Lewy bodies. However, no association was observed between immunostaining and either DNA breaks or activated caspase-3. In an analysis of 4 PD brains, Jellinger (2000) did not observe any difference in the expression of c-jun as compared to controls.

2.5. Mitogen-activated protein kinase signaling: lessons from a clinical trial in Parkinson's disease

The preclinical data indicating that CEP1347 is neuroprotective in a variety of PD models, particularly the MPTP primate model, in conjunction with proven safety and tolerability in PD patients (Parkinson Study Group, 2004), provided a sound basis for a Phase II/III trial of its ability to provide neuroprotection. This trial, named PRECEPT, was carried out with 806 early PD patients not yet requiring dopaminergic therapy (presented as a Late Breaking Abstract, I. Shoulson for the Parkinson Study Group, Annual Meeting of the American Academy of Neurology, 2006). It was a double-blind, prospective comparison of placebo to 3 doses of CEP1347: 10, 25 and 50 mg bid, utilizing an endpoint of disability requiring levodopa therapy. The study was terminated early after an average of 21.4 months of follow-up when a planned interim analysis demonstrated that it would be futile to continue treatment. At that time, 57% of patients on placebo had reached endpoint, whereas 65%, 59% and 64% of those randomized to 10, 25, and 50 mg bid, respectively, had reached endpoint. These disappointing negative results call for a critical reassessment of the hypotheses and preclinical experimental data which provided the scientific rationale for the trial. Based on the trial's intent, what appears at first glance to be the most straightforward, and the most disappointing, set of conclusions would be that in spite of effective inhibition of the MLK in diseased dopamine neurons, PD patients continued to progress. If we propose that progression of the disease is directly related to dopamine neuron loss, then we can conclude that the MLK do not play a role in dopamine neuron death in PD, the preclinical data notwithstanding.

However, this set of conclusions cannot be drawn from the negative results of the trial, based on considerations related to its implementation and our current state of knowledge of CEP1347 and PD. First, it is not known whether any of the doses of CEP1347 actually achieved inhibition of MLK in the SN or in any brain region. The doses selected were based approximately on the effective doses used in the MPTP animal model studies (Waldmeier et al., 2006). However, in the primate preclinical studies, it is not known if MLK inhibition was achieved in brain. Even if we accept, without evidence, that MLK inhibition was achieved, it is unknown whether the drug or any of its metabolites have other effects which may impact a possible response to MLK inhibition when administered to human patients.

If we ignore the above concerns and assume that in the trial CEP1347 provided in brain an effective and selective inhibition of the MLK, which did not prevent progression of parkinsonism, we still would not be able to conclude that it failed to abrogate dopamine neuron death, for the reason that we do not know the precise relationship between ongoing dopamine neuron loss and the progression of Parkinson signs. This relationship is unlikely to be a simple one. Parkinson signs do not appear until about 50% of SN dopamine neurons are lost (Marsden, 1990). It is likely that clinical signs do not appear with early lower levels of neuron loss because there are compensatory changes in the nigrostriatal system at the striatal dopaminergic terminal level, as described in experimental models of parkinsonism (Zigmond et al., 1990). These neurochemical changes include increased synthesis and release of dopamine, diminished reuptake, and the development of postsynaptic receptor supersensitivity. In addition to these neurochemical changes to compensate for dopamine neuron loss, there are likely to be anatomical responses, such as regeneration of damaged axons. These compensatory changes are likely to provide a "buffer" between neuron loss and the eventual appearance of clinical signs. Therefore, the appearance of worsening Parkinson signs with increasing disease duration may, within a limited timeframe, not be due to neuronal loss, but instead due to failure of these compensatory process, or cellular dysfunction resulting in diminished phenotype (Hirsch et al., 1988), or axonal degeneration.

If, in spite of these considerations, if we accept for the sake of discussion that progression of clinical signs may be directly related to the loss of SN neurons, it would remain difficult within the timeframe of the trial to detect protection. Based on the work of Fearnley and Lees (1991), about 45% total SN pigmented neurons are lost within the first decade with an exponential time course; interpolation of their data suggests that about a 15% loss would take place within the 21 month timeframe of the PRECEPT trial early in the course of the disease. If MLK inhibition blocks 50% of neuron death, it would be a substantial effect. But within this timeframe, this magnitude of effect would only reduce a 15% loss to a 7.5% loss. Thus, a substantial protective effect would have a minimal

impact on the numbers of neurons lost and may not be detectable by assessment of clinical signs.

It is important for this discussion to realize that the preclinical studies of the MLK and JNK signaling cascade principally demonstrated neuroprotection at the cell body level. In these studies, abrogation of these cascades blocked PCD and the resulting cell soma destruction. While some studies demonstrated axonal protection, it is now known that the pathways of axonal destruction are likely to be different from those of PCD (Raff et al., 2002), and the precise role, if any, of MLK–JNK signaling in axonal degeneration is unknown.

Therefore, although the outcome of the PRECEPT trial is a disappointment, it should not lead us to the premature conclusions that the JNK cascade, or PCD altogether, are irrelevant to neuron death in PD. Nor can it be taken as definitive evidence that MLK inhibition is a failed strategy. Some of the limitations on interpretation of the PRECEPT trial related to our lack of understanding of the neural basis of progression of PD, particularly our minimal understanding of the relationship between neuron loss and progression, apply, of course, not only to the PRECEPT trial, but also to other trials of neuroprotective approaches.

2.6. Mutations in leucine-rich repeat kinase 2 cause an autosomal dominant form of PD

Mutations in the gene *leucine-rich repeat kinase 2* (*LRRK2*, the protein product is also known as dardarin; Paisan-Ruiz et al., 2004; Zimprich et al., 2004)are an important cause of familial and some sporadic cases of PD. Mutations in LRRK2 cause an autosomal dominant form of PD, and they have attracted great interest because, unlike mutations in α-synuclein, which also cause autosomal dominant PD, they are common, particularly in select ethnic groups (Lesage et al., 2006; Ozelius et al., 2006). The *LRRK2* gene encodes a 2527-amino acid protein which contains several domains, beginning from the N-terminal: the leucine-rich repeat region, a Ras of complex proteins (Roc) domain belonging to the Ras/GTPase superfamily, a C terminal of Roc (COR) domain, a nonreceptor tyrosine kinase-like domain, and a WD40 domain. The kinase domain most closely resembles kinases of the MLK family, and LRRK2 has been demonstrated to have MLK-like activity (West et al., 2005; reviewed in (Mata et al., 2006)).

West et al. (2005) have demonstrated, by autophosphorylation assay, that both the G2019S and R1441C mutations associated with PD increase LRRK2 kinase activity. This observation is of great interest, given the known role of the MLK in mediating PCD, as discussed above. Smith et al. (2005) have shown that mutant forms of LRRK2 are toxic to cells, both human SH-SY5Y cells and mouse primary cortical neurons, and induce death by apoptosis. Greggio et al. (2006) likewise observed an increase in the kinase activity of the G2019S mutant, but not in the R1441C or Y1699C mutations. They observed 2 phenotypes in transiently transfected cells: the formation of cellular inclusions and the induction of cell death. Using wildtype and mutant kinase dead constructs, they demonstrated that both of these phenotypes depend on kinase activity. Smith et al. (2006) have likewise demonstrated that cellular toxicity depends on kinase activity. In addition, they showed that the kinase domain is regulated by GTP interaction with the GTPase Roc domain. Gloeckner et al. (2006) demonstrated a modest increase in kinase activity of the I2020T mutant. They further demonstrated that, like other MLK, LRRK2 molecules are capable of dimerization and autophosphorylation. Investigation of the LRRK2 homologue LRRK1 confirms that it is an active kinase, and that it is also regulated by GTP binding within the Roc domain (Korr et al., 2006). However, when the PD-causing mutations of LRRK2 are incorporated into the sequence of LRRK1, they result in decreased kinase activity. Thus, the precise role of the LRRK2 kinase activity in pathogenesis of PD will require further investigation, but it clearly has the potential to become a therapeutic target.

3. The Akt signaling pathway and neuron survival

3.1. Overview of Akt signaling

Akt is a serine/threonine kinase with diverse roles related to the regulation of cell growth, proliferation, migration, glucose metabolism, transcription, protein synthesis, angiogenesis and cell survival (Brazil & Hemmings, 2001). For the purpose of this review, we will focus only on its role as a mediator of cell survival by inhibition of apoptosis; for more general treatments, the reader is referred to excellent reviews (Brazil & Hemmings, 2001; Vivanco & Sawyers, 2002; Brazil et al., 2004). The discovery of Akt began in 1977 with the isolation of transforming murine leukemia virus, termed AKT8, by Staal et al. (1977). Subsequently, in 1991 the oncogene encoded by the virus, v-akt, was cloned (Bellacosa et al., 1991). The same gene was cloned independently that year by 2 other groups and referred to as “related to A and C kinases” (RAC) and PKB (Coffer and Woodgett, 1991; Jones et al., 1991). There are now known to be 3 isoforms of Akt, encoded by 3 separate genes in mammalian cells: Akt1/PKBα, Akt2/PKBβ, Akt3/PKBγ (Fig. 3). All 3 isoforms are widely expressed, but Akt1 is predominant in most tissues (Kandel & Hay, 1999). The Akts are in the family of the AGC kinases (PKA, PKG, and PKC related kinases), which are characterized by a central kinase domain and a C-terminal hydrophobic motif (see Fig. 3).

Activation of Akt occurs following the binding of a protein growth factor to its receptor on the surface of the cell (see Fig. 4). Ligand binding induces autophosphorylation of tyrosine residues in the cytoplasmic portion of the receptor, resulting in the recruitment and activation of phosphatidylinositol 3-kinase (PI3K). PI3K phosphorylates phosphatidylinositol 4,5 biphosphate (PtdIns(4,5)P2) to phosphatidylonositol 3,4,5 triphosphate (PtdIns (3,4,5)P3), which mediates localization of Akt to the inner surface of the cell membrane by interaction with its pleckstrin homology domain (PH). PtdIns(3,4,5)P3 can be dephosphorylated by phosphatase and tensin homologue deleted on chromosome ten (PTEN), which thus serves as a negative regulator of Akt activation. Once localized to the inner surface of the cell membrane, Akt is activated by phosphorylation at 2

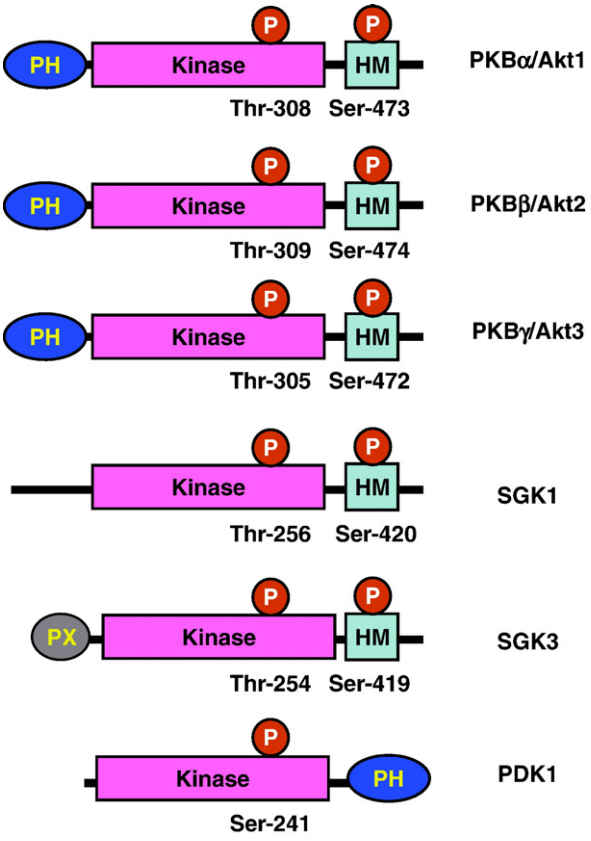

Fig. 3. The domain structure of the AGC kinases. The protein isoforms of Akt, and other AGC kinases, have a central kinase domain, and a C-terminal hydrophobic domain. Abbreviations: HM: hydrophobic motif, SGK1: serum and glucocorticoid induced protein kinase. Adapted from Hanada et al. (2004), with permission.

critical residues: Thr 308 in the kinase domain and Ser473 in the hydrophobic motif. The Thr308 kinase is 3-phosphoinsitide-dependent kinase 1 (PDK1); like Akt it is localized to the inner surface of the cell membrane by an interaction between PtdIns (3,4,5)P3 and its PH (Hanada et al., 2004). Like Akt, PDK1 is in the family of AGC kinases (Hanada et al., 2004; Fig. 3). The kinase for the Ser473 residue in the hydrophobic motif had been elusive and the subject of debate for many years, but recently Sarbassov and colleagues have provided compelling evidence that it is a complex consisting of mammalian target of rapamycin (mTOR), G-protein ß-subunit-like protein (GβL) and rictor (Sarbassov et al., 2005; Bayascas & Alessi, 2005).

The first evidence that PI3K/AKT signaling plays a role in supporting the survival of neurons was obtained in studies of NGF-treated PC12 cells (Yao & Cooper, 1995). Yao and Cooper demonstrated that the ability of NGF to block apoptosis in these cells was abrogated by treatment with 2 specific inhibitors of PI3K. In addition they showed that the ability of platelet-derived growth factor (PDGF) to prevent apoptosis was blocked in cells expressing a mutant PDGF receptor which failed to activate PI3K (Yao & Cooper, 1995). Subsequently, other investigators confirmed that PI3K signaling could prevent cell death in a variety of other tissue culture models utilizing cerebellar, sympathetic (Crowder & Freeman, 1998), sensory, cortical and motor neurons (reviewed in Kaplan & Miller, 2000). A role for Akt in mediating neuronal survival was first demonstrated by Dudek et al. (1997) in a primary postnatal cerebellar granule cell culture model, in which apoptosis is induced by either low potassium or growth factor withdrawal (D'Mello et al., 1993). Dudek et al. (1997) demonstrated that transfection of these neurons with either of 2 dominant negative forms of Akt blocked the ability of insulin to promote survival, which they showed was mediated by activation of PI3K. They further demonstrated that survival was enhanced by transfection with wild-type Akt. Since these initial observations, a large number of studies have demonstrated that Akt protects from apoptosis due to a wide variety of death-inducing stimuli, including the withdrawal of growth factors, UV irradiation, matrix detachment, cell cycle disturbance, DNA damage, and treatment of cells with anti-Fas antibody (reviewed in Datta et al., 1999). Conversely, Luo et al. (2003) have shown that in a variety of tissue culture models, deactivation of Akt accompanies cell death induced by many different agents.

3.2. Inhibition of apoptosis by Akt in vitro

Akt has been demonstrated to inhibit apoptosis by many mechanisms affecting diverse apoptotic pathways at multiple levels, from upstream signaling pathways which regulate transcriptional activity, to downstream targets, such as caspase-9 (Fig. 5). It is beyond the scope of the present review to consider all of these mechanisms in detail. For a more thorough overview, the reader is referred to several comprehensive reviews (Datta et al., 1999; Brunet et al., 2001; Downward, 2004). For the purposes of this review, we will illustrate some of the mechanisms by which Akt acts to inhibit pro-apoptotic transcriptional activation by c-jun and the forkhead family of transcription factors. In addition, we will briefly outline the mechanism by which Akt activates eIF-4E in a recently defined, novel role as an inhibitor of apoptosis by regulating mitochondrial cytochrome c release.

Akt negatively regulates the phosphorylation and activation of c-jun by a number of mechanisms (Fig. 5). As previously discussed in relation to MLK-JNK signaling, in some cellular contexts, MAPKKK are activated by the small GTP binding proteins Rac1 or Cdc42 (see Fig. 1). Both of these signaling proteins have been shown to participate in NGF withdrawal-induced apoptosis in sympathetic neurons (Bazenet et al., 1998) and in PC12 cells (Xu et al., 2001). The relevance of Cdc42 signaling to the death of dopamine neurons in vivo is supported by the demonstration in an axotomy model that transduction of these neurons with a dominant negative form protects them from neurodegeneration (Crocker et al., 2006). Akt has been shown to phosphorylate Rac1 at serine 71, and thereby reduce its ability to bind GTP, as required for activation (Kwon et al., 2000).

Downstream of Rac1 and Cdc42, Akt also negatively regulates MLK3. Barthwal et al. (2003) have demonstrated that Akt interacts directly with MLK3, via a C-terminal domain. Within this domain, Akt phosphorylates serine 674, resulting in diminished JNK activation by MLK3, and decreased cell death. In the model examined, the ability of insulin to attenuate

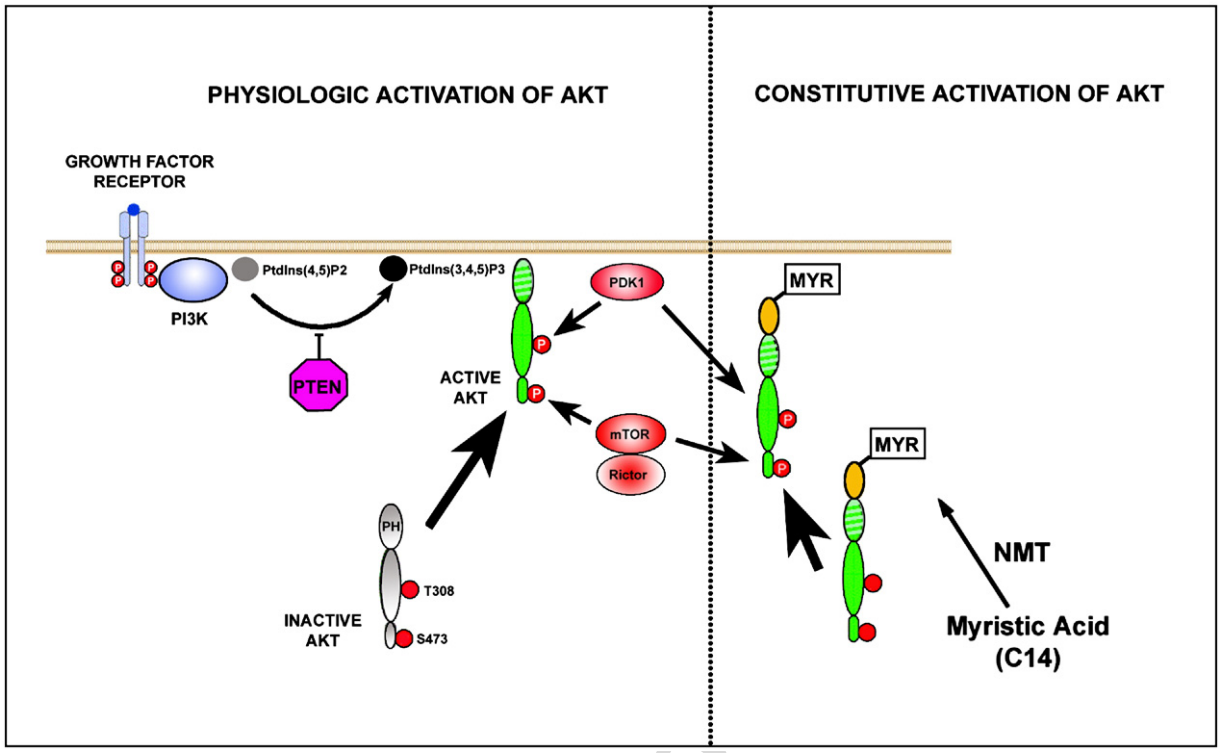

Fig. 4. Akt signaling pathways. The physiologic pathways for the activation of Akt, as described in the text, are shown on the left side of the image. On the right side are shown the pathways for constitutive activation of Akt following N-terminal incorporation of a myristoylation signal (yellow oval). The myristoylation signal is targeted by *N*-myristoyltransferase (NMT) for the post-translational transfer of myristate onto the N-terminal glycine of the modified protein. Myristic acid is a C14 saturated fatty acid which targets the protein to the inner surface of the cell membrane, where it is phosphorylated, even in the absence of PI3K activation. This myristoylated form of Akt was used in our in vivo studies on SN dopamine neurons, described in the text (Ries et al., 2006).

MLK3, MKK7 and JNK activation was dependent on PI3K. Thus, in this system, the prosurvival effects of insulin appear to be mediated by PIK3/AKT signaling, and dependent on Akt phosphorylation of MLK3. Further downstream of the MLK, Akt also interacts with and phosphorylates MKK4 at serine 78 (Park et al., 2002). Phosphorylation at this residue inhibits MKK4-mediated apoptosis.

MLK3 is not the only MAPKKK target of Akt. ASK1 is a MAPKKK which activates both JNK and p38 (Ichijo et al., 1997). Kim et al. (2001) have demonstrated that Akt binds to ASK1, phosphorylates it at serine 83, and thereby reduces its kinase activity. This modification of ASK1 results in reduced activation of JNK, and a reduction of apoptosis in cell lines.

The scaffold proteins JIP1 and POSH, described earlier, are also Akt targets. Akt1 binds to JIP1 in primary neurons and inhibits its ability to potentiate JNK activation (Kim et al., 2002). Similarly, Figueroa et al. (2003) have shown that Akt2 binds to POSH and negatively regulates its ability to activate JNK. This inhibition appears to be mediated by phosphorylation of MLK3, resulting in its dissociation from the POSH signaling complex.

Whereas Akt inhibition of c-jun activation is achieved by phosphorylation of these diverse upstream activators, its inhibition of the forkhead box O (FoxO) family of transcription factors is achieved by direct interaction with, and phosphorylation of, the proteins (Fig. 5). The FoxO family of transcription factors plays a role in diverse cellular functions, including initiation of apoptosis by the induction of the proapoptotic genes FasL (Brunet et al., 1999) and Bim (Dijkers et al., 2000), (reviewed in van der Heide et al., 2006). To date, 4 members of the FoxO family have been identified in mammalian cells: FoxO1, FoxO3, FoxO4, and FoxO6. All but FoxO6 are expressed in brain. The FoxO all contain 3 Akt phosphorylation motifs. Brunet et al. (1999) demonstrated that Akt phosphorylates FoxO3 (also known as FKHRL1) at T32, S253 and S315 in cells, which enables an interaction with protein 14-3-3. This interaction sequesters FoxO3 in the cytoplasm, preventing it from migrating to the nucleus and activating proapoptotic gene transcription. The critical role of phosphorylation of these residues in FoxO3 to abrogate its proapoptotic effects was confirmed by creating a triple mutant form in which nonphosphorylatable alanines were substituted. The triple mutant form of FoxO3 translocated to the nucleus and induced apoptosis even in the presence of the survival factor IGF1 (Brunet et al., 1999).

Akt also inhibits apoptosis at the post-transcriptional level by phosphorylation of pro-apoptotic proteins, including premitochondrial mediators, such as Bad (Datta et al., 1997) and postmitochondrial mediators such as caspase-9 (Zhou et al., 2000). More recently, an antiapoptotic effect of Akt has been demonstrated to be mediated through activation of mTor (Wendel et al., 2004; McCormick, 2004). Wendel and colleagues have demonstrated in a murine lymphoma model that the antiapoptotic effects of Akt can be blocked by rapamycin, an

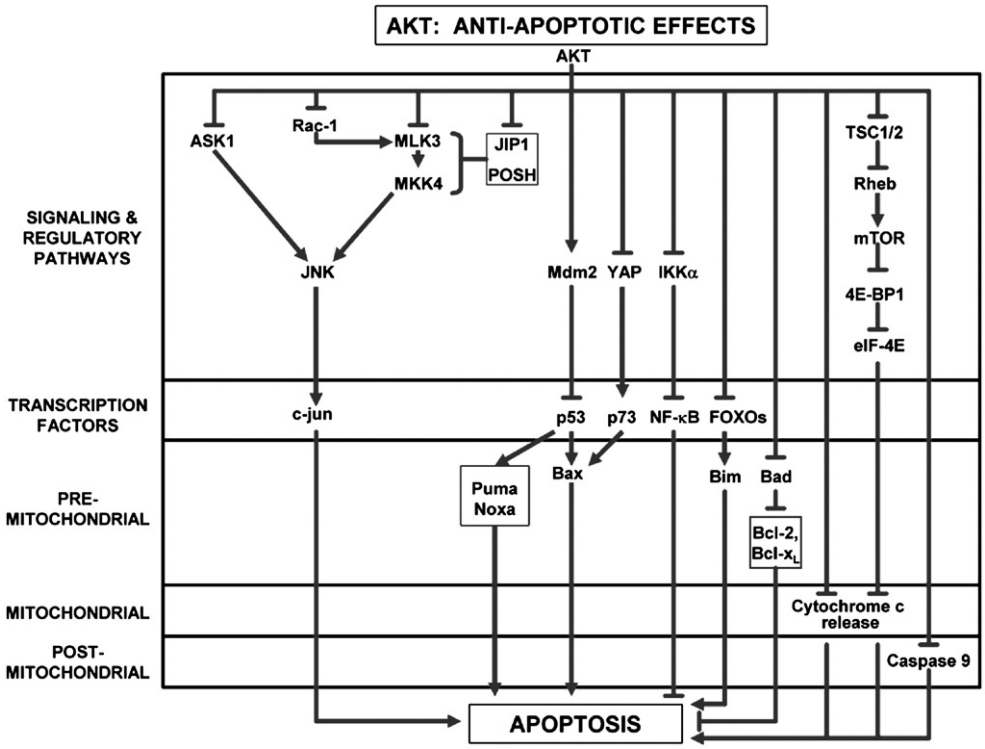

Fig. 5. Antiapoptotic mechanisms of Akt. Akt has been described to inhibit apoptosis at multiple levels: signaling pathways, transcription factors, and, in the intrinsic pathway of PCD, at pre-mitochondrial, mitochondrial, and post-mitochondrial levels. Detailed mechanisms for some of these pathways are presented in the text. For more complete reviews, see (Datta et al., 1999; Brunet et al., 2001; Downward, 2004).

inhibitor of mTor. The antiapoptotic effect of Akt could be restored by eIF-4E, and this effect was not blocked by rapamycin, indicating that eIF-4E is downstream. While eIF-4E is best known for its role as an elongation initiation factor for the translation of protein from mRNA, it has more recently been shown to play a role in blocking apoptosis as well. Li et al. (2003) have demonstrated that eIF-4E is capable of blocking apoptosis induced by c-myc. They show that eIF-4E increases both the abundance and translation of the mRNA of the anti-apoptotic Bcl-X_L, and thereby decreases mitochondrial cytochrome *c* release (Li et al., 2003).

Activation of eIF-4E by mTor is mediated by phosphorylation and inhibition of 4E-BP1, a negative regulator of eIF-4E (reviewed in Schmelzle & Hall, 2000; Manning & Cantley, 2003). Activation of mTor by Akt is achieved indirectly by Akt phosphorylation and inhibition of tuberin (tuberous sclerosis complex [TSC] 2). Tuberin/TSC2 functions in a complex with hamartin/TSC1 as a GTPase-activating protein (GAP) to inhibit a Ras-related small GTPase Rheb (Manning & Cantley, 2003). Rheb is positive regulator of Tor signaling. Thus, Akt ultimately activates mTor by blocking negative regulation of Rheb (Manning & Cantley, 2003).

3.3. Effects of Akt in vivo

In spite of the extensive information available about the mechanisms of the antiapoptotic effects of Akt derived from studies in tissue culture, there have been remarkably few studies of its effects on neurons in vivo. In one promising study, Namikawa et al. (2000) demonstrated in a postnatal axotomy model that transduction of motor neurons with a constitutively active form of Akt improved their survival and accelerated the regeneration of their axons.

We have recently demonstrated that a constitutively active, myristoylated form of Akt (Myr-Akt) has potent anti-apoptotic effects on dopamine neurons of the SN in vivo. Transduction of these neurons by use of an adeno-associated virus (AAV) vector with Myr-Akt inhibited apoptosis by about 80% in an intrastriatal 6OHDA model of parkinsonism (Ries et al., 2006). This inhibition of dopamine neuron death resulted in an almost complete preservation of these neurons; in the presence of AAV Myr-Akt there was only a 13% (not significant) loss, whereas in AAV-GFP controls, there was an 80% loss, typical of this model. AAV-Myr-Akt was able to protect dopamine neurons in this model not only when it was administered before 6OHDA, but also when it was administered 3 weeks after. In this delayed delivery paradigm, only 14% of dopamine neurons survived in AAV GFP controls, whereas 50% survived in the AAV Myr-Akt group.

Many antiapoptotic approaches have been demonstrated to successfully protect neuronal cell bodies, but unfortunately they often do not preserve axons (Eberhardt et al., 2000; Silva et al., 2005). Myr-Akt, however, not only preserved neuronal cells bodies, but also dopaminergic striatal projections (Ries et al., 2006). Following 6OHDA lesion, striatal dopaminergic innervation was reduced by 74% in control mice treated with AAV GFP, whereas in mice treated with AAV Myr-GFP, it was reduced by only 26%. Even when AAV Myr-Akt was injected

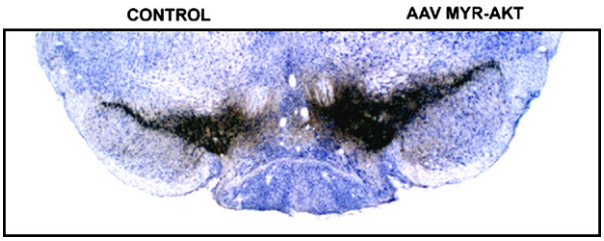

Fig. 6. Effect of AAV Myr-Akt on SN dopaminergic neurons in a 22 month old mouse. Aged mice were injected into the SN with either AAV GFP or AAV Myr-Akt and sacrificed for morphologic analysis 7 weeks later. Myr-Akt induced a 31% increase in the total volume of the SN as compared to the contralateral non-injected SN. There was also an increase in the size of individual TH-positive neurons. Myr-Akt also induced a vigorous sprouting response in aged mice, with a 37% increase in striatal TH optical density values on the AAV Myr-Akt injected side in comparison to contralateral control and both striata of AAV GFP injected mice (not shown).

into the SN 3 weeks after 6OHDA, it was still highly protective of striatal dopaminergic fibers. In AAV GFP-treated control mice, fiber density was reduced by 90%; whereas in AAV-Myr-Akt-treated mice, it was reduced only 50%. This morphologic preservation of striatal dopaminergic innervation was associated with preserved striatal dopaminergic biochemical indices and behavioral recovery (Ries et al., 2006).

In addition to these striking neuroprotective effects in the context of a neurotoxin lesion, Myr-Akt also had remarkable trophic effects on unlesioned dopamine neurons in normal adult and aged mice. Neurotrophic effects included an increase in the size of individual dopamine neurons, the regional volume of the SNpc, and an increase in the expression of the phenotypic protein TH, associated with an increased abundance of dopamine. In addition, there was an induction of sprouting into the striatum, with an associated increase in striatal dopamine turnover and an augmented response to amphetamine (Ries et al., 2006). Neurotrophic effects on dopamine neurons of the SNpc in aged mice are illustrated Fig. 6.

4. Conclusions

As reviewed herein, there is an abundance of evidence derived from both in vitro and in vivo studies to suggest that MAPK signaling pathways mediate PCD in neurons, including dopamine neurons, and that inhibition of them can abrogate neuron death. In spite of this compelling pre-clinical evidence, and a well-powered phase II/III clinical trial, an MLK inhibitor failed to provide neuroprotection in PD. As disappointing as this result is, it should not lead as to the conclusion that this is a failed approach, for the simple reason that we do not know if kinase inhibition was achieved in brain. Nevertheless, the trial does suggest important lessons, and it does encourage a more critical analysis of our goals in providing neuroprotection. We know that CEP1347 was very effective in preventing progression of parkinson signs in MPTP-treated primates. The negative results of the trial may therefore suggest that this neurotoxin model does not provide a reliable prediction of neuroprotective efficacy.

The aforementioned preclinical data in almost all instances investigated the ability of MAPK inhibition to prevent neuron cell body death as the primary outcome measure. In the clinical trial, in which the primary outcome measure was the progression of parkinsonian signs, it was assumed that prevention of neuron death, as demonstrated in the preclinical studies, would translate into a slowing of disease progression in the clinical context. But this assumption is unlikely to be true; we know that extensive neuron loss occurs prior to the appearance of any Parkinson signs. While at some level, there is likely to ultimately be a relationship between dopamine neuron loss and clinical progression, the relationship is unlikely to be simple linear one, and it may change over the course of the disease. It is, for example, possible that after the disease is first diagnosed, over the course of the first year or 2 (the period which was the subject of the PRECEPT trial), the major determinant of progression is axonal degeneration or regenerative failure, and not neuron loss.

Another possible lesson of the PRECEPT trial is that it may be insufficient to attempt to inhibit only one pathway of PCD. There is such diversity and redundancy in these pathways that the only approaches likely to be effective will simultaneously target many of them. An example of such an approach is the use of the survival signaling kinase Akt, which shows initial promise in rodent studies.

Whether or not medical or gene therapy approaches to inhibition of MAPK signaling ever prove useful as therapies for neuroprotection in PD, the discovery of disease-causing mutations in the MLK-like kinase LRRK2 clearly indicates the importance of these related pathways in regulating the viability of adult dopamine neurons. Thus, these pathways are exceedingly likely to yield important future therapeutic targets.

Acknowledgments

The author is supported by NIH grants NS26836, NS38370, Department of Defense grant DAMD17-03-1-0492, Parkinson's Disease Foundation, Lowenstein Foundation, and the Michael J Fox Foundation.

References

Anderson, A. J., Cummings, B. J., & Cotman, C. W. (1994). Increased immunoreactivity for Jun- and Fos-related proteins in Alzheimer's disease: association with pathology. *Exp Neurol 125*, 286−295.

Anderson, A. J., Su, J. H., & Cotman, C. W. (1996). DNA damage and apoptosis in Alzheimer's disease: colocalization with c-Jun immunoreactivity, relationship to brain area, and effect of postmortem delay. *J Neurosci 16*, 1710−1719.

Barthwal, M. K., Sathyanarayana, P., Kundu, C. N., Rana, B., Pradeep, A., Sharma, C., et al. (2003). Negative regulation of mixed lineage kinase 3 by protein kinase B/AKT leads to cell survival. *J Biol Chem 278*, 3897−3902.

Bayascas, J. R., & Alessi, D. R. (2005). Regulation of Akt/PKB Ser473 phosphorylation. *Mol Cell 18*, 143−145.

Bazenet, C. E., Mota, M. A., & Rubin, L. L. (1998). The small GTP-binding protein Cdc42 is required for nerve growth factor withdrawal-induced neuronal death. *Proc Natl Acad Sci U S A 95*, 3984−3989.

Behrens, A., Sibilia, M., & Wagner, E. F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet 21*, 326−329.

Bellacosa, A., Testa, J. R., Staal, S. P., & Tsichlis, P. N. (1991). A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science 254*, 274−277.

Blennow, K., de Leon, M. J., & Zetterberg, H. (2006). Alzheimer's disease. *Lancet 368*, 387−403.

Bower, J. H., Maraganore, D. M., McDonnell, S. K., & Rocca, W. A. (2000). Influence of strict, intermediate, and broad diagnostic criteria on the age- and sex-specific incidence of Parkinson's disease. *Movement Disorders 15*, 819−825.

Brazil, D. P., & Hemmings, B. A. (2001). Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci 26*, 657−664.

Brazil, D. P., Yang, Z. Z., & Hemmings, B. A. (2004). Advances in protein kinase B signalling: AKTion on multiple fronts. *Trends Biochem Sci 29*, 233−242.

Bredesen, D. E., Rao, R. V., & Mehlen, P. (2006). Cell death in the nervous system. *Nature 443*, 796−802.

Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., et al. (1999). Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell 96*, 857−868.

Brunet, A., Datta, S. R., & Greenberg, M. E. (2001). Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. *Curr Opin Neurobiol 11*, 297−305.

Choi, H. K., Won, L. A., Kontur, P. J., Hammond, D. N., Fox, A. P., Wainer, B. H., et al. (1991). Immortalization of embryonic mesencephalic dopaminergic neurons by somatic cell fusion. *Brain Res 552*, 67−76.

Coffer, P. J., & Woodgett, J. R. (1991). Molecular cloning and characterisation of a novel putative protein-serine kinase related to the cAMP-dependent and protein kinase C families. *Eur J Biochem 201*, 475−481.

Cookson, M. R. (2005). The biochemistry of Parkinson's disease. *Annu Rev Biochem 74*, 29−52.

Crocker, S. J., Lamba, W. R., Smith, P. D., Callaghan, S. M., Slack, R. S., Anisman, H., et al. (2001). c-Jun mediates axotomy-induced dopamine neuron death in vivo. *Proc Natl Acad Sci U S A 98*, 13385−13390.

Crocker, S. J., Hayley, S. P., Smith, P. D., Mount, M. P., Lamba, W. R., Callaghan, S. M., et al. (2006). Regulation of axotomy-induced dopaminergic neuron death and c-Jun phosphorylation by targeted inhibition of cdc42 or mixed lineage kinase. *J Neurochem 96*, 489−499.

Crowder, R. J., & Freeman, R. S. (1998). Phosphatidylinositol 3-kinase and Akt protein kinase are necessary and sufficient for the survival of nerve growth factor-dependent sympathetic neurons. *J Neurosci 18*, 2933−2943.

D'Mello, S. R., Galli, C., Ciotti, T., & Calissano, P. (1993). Induction of apoptosis in cerebellar granule neurons by low potassium: inhibition of death by insulin-like growth factor I and cAMP. *Proc Natl Acad Sci U S A 90*, 10989−10993.

Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., & Gotoh, Y. (1997). Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell 91*, 231−241.

Datta, S. R., Brunet, A., & Greenberg, M. E. (1999). Cellular survival: a play in three Akts. *Genes Dev 13*, 2905−2927.

Davis, R. J. (1994). MAPKs: new JNK expands the group. *TIBS 19*, 470−473.

Davis, R. J. (2000). Signal transduction by the JNK group of MAP kinases. *Cell 103*, 239−252.

Deckwerth, T. L., & Johnson, E. M. (1993). Temporal analysis of events associated with programmed cell death (apoptosis) of sympathetic neurons deprived of nerve growth factor. *J Cell Biol 123*, 1207−1222.

Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., & Deng, T. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell 76*, 1025−1037.

Dickens, M., Rogers, J. S., Cavanagh, J., Raitano, A., Xia, Z., & Halpern, J. R. (1997). A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science 277*, 693−696.

Dijkers, P. F., Medema, R. H., Lammers, J. W., Koenderman, L., & Coffer, P. J. (2000). Expression of the pro-apoptotic Bcl-2 family member Bim is regulated by the forkhead transcription factor FKHR-L1. *Curr Biol 10*, 1201−1204.

Donovan, N., Becker, E. B., Konishi, Y., & Bonni, A. (2002). JNK phosphorylation and activation of BAD couples the stress-activated signaling pathway to the cell death machinery. *J Biol Chem 277*, 40944−40949.

Downward, J. (2004). PI 3-kinase, Akt and cell survival. *Semin Cell Dev Biol 15*, 177−182.

Dragunow, M., Young, D., Hughes, P., MacGibbon, G., Lawlor, P., Singleton, K., et al. (1993). Is c-Jun involved in nerve cell death following status epilepticus and hypoxic-ischaemic brain injury? *Mol Brain Res 18*, 347−352.

Dragunow, M., Beilharz, E., Sirimanne, E., Lawlor, P., Williams, C., Bravo, R., et al. (1994). Immediate-early gene protein expression in neurons undergoing delayed death, but not necrosis, following hypoxic-ischaemic injury to the young rat brain. *Mol Brain Res 25*, 19−33.

Dudek, H., Datta, S. R., Franke, T. F., Birnbaum, M. J., Yao, R., Cooper, G. M., et al. (1997). Regulation of neuronal survival by the serine-threonine protein kinase Akt. *Science 275*, 661−665.

Eberhardt, O., Coelln, R. V., Kugler, S., Lindenau, J., Rathke-Hartlieb, S., Gerhardt, E., et al. (2000). Protection by synergistic effects of adenovirus-mediated X-chromosome-linked inhibitor of apoptosis and glial cell line-derived neurotrophic factor gene transfer in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of Parkinson's disease. *J Neurosci 20*, 9126−9134.

Edwards, S. N., & Tolkovsky, A. M. (1994). Characterization of apoptosis in cultured rat sympathetic neurons after nerve growth factor withdrawal. *J Cell Biol 124*, 537−546.

Eilers, A., Whitfield, J., Babij, C., Rubin, L. L., & Ham, J. (1998). Role of the Jun kinase pathway in the regulation of c-Jun expression and apoptosis in sympathetic neurons. *J Neurosci 18*, 1713−1724.

Ellis, R. E., Yuan, J., & Horvitz, H. R. (1991). Mechanisms and functions of cell death. *Annu Rev Cell Biol 7*, 663−698.

Estus, S., Zaks, W. J., Freeman, R. S., Gruda, M., Bravo, R., & Johnson, E. M. (1994). Altered gene expression in neurons during programmed cell death identification of c-jun as necessary for neuronal apoptosis. *J Cell Biol 127*, 1717−1727.

Fearnley, J. M., & Lees, A. J. (1991). Ageing and Parkinson's disease: substantia nigra regional selectivity. *Brain 114*, 2283−2301.

Ferrer, I., Olive, M., Blanco, R., Cinos, C., & Planas, A. M. (1996a). Selective c-Jun overexpression is associated with ionizing radiation-induced apoptosis in the developing cerebellum of the rat. *Mol Brain Res 38*, 91−100.

Ferrer, I., Olive, M., Ribera, J., & Planas, A. M. (1996b). Naturally occurring (programmed) and radiation-induced apoptosis are associated with selective c-jun expression in the developing rat brain. *Eur J Neurosci 8*, 1286−1298.

Ferrer, I., Blanco, R., Carmona, M., Puig, B., Barrachina, M., & Gomez, C. (2001). Active, phosphorylation-dependent mitogen-activated protein kinase (MAPK/ERK), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38 kinase expression in Parkinson's disease and dementia with Lewy bodies. *J Neural Transm 108*, 1383−1396.

Figueroa, C., Tarras, S., Taylor, J., & Vojtek, A. B. (2003). Akt2 negatively regulates assembly of the POSH-MLK-JNK signaling complex. *J Biol Chem 278*, 47922−47927.

Furuya, T., Hayakawa, H., Yamada, M., Yoshimi, K., Hisahara, S., Miura, M., et al. (2004). Caspase-11 mediates inflammatory dopaminergic cell death in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *J Neurosci 24*, 1865−1872.

Gallo, K. A., & Johnson, G. L. (2002). Mixed-lineage kinase control of JNK and p38 MAPK pathways. *Nat Rev Mol Cell Biol 3*, 663−672.

Ganguly, A., Oo, T. F., Rzhetskaya, M., Pratt, R., Yarygina, O., Momoi, T., et al. (2004). CEP11004, a novel inhibitor of the mixed lineage kinases, suppresses apoptotic death in dopamine neurons of the substantia nigra induced by 6-hydroxydopamine. *J Neurochem 88*, 469−480.

Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol 119*, 493−501.

Glicksman, M. A., Chiu, A. Y., Dionne, C. A., Harty, M., Kaneko, M., Murakata, C., et al. (1998). CEP-1347/KT7515 prevents motor neuronal programmed cell death and injury-induced dedifferentiation in vivo. *J Neurobiol 35*, 361−370.

Gloeckner, C. J., Kinkl, N., Schumacher, A., Braun, R. J., O'Neill, E., Meitinger, T., et al. (2006). The Parkinson disease causing LRRK2 mutation I2020T is associated with increased kinase activity. *Hum Mol Genet 15*, 223−232.

Greggio, E., Jain, S., Kingsbury, A., Bandopadhyay, R., Lewis, P., Kaganovich, A., et al. (2006). Kinase activity is required for the toxic effects of mutant LRRK2/dardarin. *Neurobiol Dis 23*, 329−341.

Gubits, R. M., Burke, R. E., Casey McIntosh, G., Bandele, A., & Munell, F. (1993). Immediate early gene induction after neonatal hypoxia-ischemia. *Brain Res Mol Brain Res 18*, 228−238.

Haas, C. A., Deller, T., Naumann, T., & Frotscher, M. (1996). Selective expression of the immediate early gene c-jun in axotomized rat medial septal neurons is not related to neuronal degeneration. *J Neurosci 16*, 1894−1903.

Ham, J., Babij, C., Whitfield, J., Pfarr, C. M., Lallemand, D., Yaniv, M., et al. (1995). A c-jun dominant negative mutant protects sympathetic neurons against programmed cell death. *Neuron 14*, 927−939.

Hanada, M., Feng, J., & Hemmings, B. A. (2004). Structure, regulation and function of PKB/AKT-a major therapeutic target. *Biochim Biophys Acta 1697*, 3−16.

Herdegen, T., Claret, F. X., Kallunki, T., Martin-Villalba, A., Winter, C., Hunter, T., et al. (1998). Lasting N-terminal phosphorylation of c-Jun and activation of c-Jun N-terminal kinases after neuronal injury. *J Neurosci 18*, 5124−5135.

Hibi, M., Lin, A., Smeal, T., Minden, A., & Karin, M. (1993). Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain. *Genes Dev 7*, 2135−2148.

Hirsch, E., Graybiel, A. M., & Agid, Y. A. (1988). Melanized dopaminergic neurons are differentially susceptible to degeneration in Parkinson's disease. *Nature 334*, 345−348.

Holtz, W. A., & O'Malley, K. L. (2003). Parkinsonian mimetics induce aspects of unfolded protein response in death of dopaminergic neurons. *J Biol Chem 278*, 19367−19377.

Hunot, S., Vila, M., Teismann, P., Davis, R. J., Hirsch, E. C., Przedborski, S., et al. (2004). JNK-mediated induction of cyclooxygenase 2 is required for neurodegeneration in a mouse model of Parkinson's disease. *Proc Natl Acad Sci U S A 101*, 665−670.

Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriguchi, T., et al. (1997). Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science 275*, 90−94.

Jackson-Lewis, V., Jakowec, M., Burke, R., & Przedborski, E. (1995). Time course and morphology of dopaminergic neuronal death caused by the neurotoxin 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine. *Neurodegeneration 4*, 257−269.

Jain, S., Wood, N. W., & Healy, D. G. (2005). Molecular genetic pathways in Parkinson's disease: a review.*Clin Sci 109*, 355−364 (Lond).

Jellinger, K. A. (2000). Cell death mechanisms in Parkinson's disease. *J Neural Transm 107*, 1−29.

Jenkins, R., & Hunt, S. P. (1991). Long-term increase in the levels of c-jun mRNA and jun protein-like immunoreactivity in motor and sensory neurons following axon damage. *Neurosci Lett 129*, 107−110.

Jenkins, R., O'Shea, R., Thomas, K. L., & Hunt, S. P. (1993). c-jun expression in substantia nigra neurons following striatal 6-hydroxydopamine lesions in the rat. *Neuroscience 53*, 447−455.

Johnson, E. M., & Deckwerth, T. L. (1993). Molecular mechanisms of developmental neuronal death. *Ann Rev Neurosci 16*, 31−46.

Jones, P. F., Jakubowicz, T., Pitossi, F. J., Maurer, F., & Hemmings, B. A. (1991). Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. *Proc Natl Acad Sci U S A 88*, 4171−4175.

Kandel, E. S., & Hay, N. (1999). The regulation and activities of the multifunctional serine/threonine kinase Akt/PKB. *Exp Cell Res 253*, 210−229.

Kaplan, D. R., & Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr Opin Neurobiol 10*, 381−391.

Kerr, J. F. R., Gobe, G. C., Winterford, C. M., & Harmon, B. V. (1995). Anatomical methods in cell death. In L. M. Schwartz, & B. A. Osborne (Eds.), *Methods in Cell Biology: Cell Death* (pp. 1−27). New York: Academic Press.

Kharbanda, S., Saxena, S., Yoshida, K., Pandey, P., Kaneki, M., Wang, Q., et al. (2000). Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. *J Biol Chem 275*, 322−327.

Kholodilov, N., Rzhetskaya, M., & Burke, R. E. (2006). Analysis of the relative abundance of the mixed lineage kinases (MLK) in human substantia nigra (SN) using real-time PCR. *Abstracts Society for Neuroscience*.

Kim, A. H., Khursigara, G., Sun, X., Franke, T. F., & Chao, M. V. (2001). Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Biol 21*, 893−901.

Kim, A. H., Yano, H., Cho, H., Meyer, D., Monks, B., Margolis, B., et al. (2002). Akt1 regulates a JNK scaffold during excitotoxic apoptosis. *Neuron 35*, 697−709.

Knusel, B., & Hefti, F. (1992). K-252 compounds: modulators of neurotrophin signal transduction. *J Neurochem 59*, 1987−1996.

Korr, D., Toschi, L., Donner, P., Pohlenz, H. D., Kreft, B., & Weiss, B. (2006). LRRK1 protein kinase activity is stimulated upon binding of GTP to its Roc domain. *Cell Signal 18*, 910−920.

Kwon, T., Kwon, D. Y., Chun, J., Kim, J. H., & Kang, S. S. (2000). Akt protein kinase inhibits Rac1-GTP binding through phosphorylation at serine 71 of Rac1. *J Biol Chem 275*, 423−428.

Kyriakis, J. M., & Avruch, J. (1990). pp54 microtubule-associated protein 2 kinase. A novel serine/threonine protein kinase regulated by phosphorylation and stimulated by poly-L-lysine. *J Biol Chem 265*, 17355−17363.

Kyriakis, J. M., & Avruch, J. (2001). Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol Rev 81*, 807−869.

Kyriakis, J. M., Banerjee, P., Nikolakaki, E., Dai, T., Rubie, E. A., Ahmad, M. F., et al. (1994). The stress-activated protein kinase subfamily of c-Jun kinases. *Nature 369*, 156−160.

Lang, A. E., & Lozano, A. M. (1998). Parkinson's disease. First of two parts. *N Engl J Med 339*, 1044−1053.

Le Niculescu, H., Bonfoco, E., Kasuya, Y., Claret, F. X., Green, D. R., & Karin, M. (1999). Withdrawal of survival factors results in activation of the JNK pathway in neuronal cells leading to Fas ligand induction and cell death. *Mol Cell Biol 19*, 751−763.

Leah, J. D., Herdegen, T., Murashov, A., Dragunow, M., & Bravo, R. (1993). Expression of immediate early gene proteins following axotomy and inhibition of axonal transport in the rat central nervous system. *Neuroscience 57*, 53−66.

Lei, K., & Davis, R. J. (2003). JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci U S A 100*, 2432−2437.

Lesage, S., Durr, A., Tazir, M., Lohmann, E., Leutenegger, A. L., Janin, S., et al. (2006). LRRK2 G2019S as a cause of Parkinson's disease in North African Arabs. *N Engl J Med 354*, 422−423.

Li, S., Takasu, T., Perlman, D. M., Peterson, M. S., Burrichter, D., Avdulov, S., et al. (2003). Translation factor eIF4E rescues cells from Myc-dependent apoptosis by inhibiting cytochrome c release. *J Biol Chem 278*, 3015−3022.

Luo, H. R., Hattori, H., Hossain, M. A., Hester, L., Huang, Y., Lee-Kwon, W., et al. (2003). Akt as a mediator of cell death. *Proc Natl Acad Sci U S A 100*, 11712−11717.

MacGibbon, G. A., Lawlor, P. A., Walton, M., Sirimanne, E., Faull, R. L., Synek, B., et al. (1997). Expression of Fos, Jun, and Krox family proteins in Alzheimer's disease. *Exp Neurol 147*, 316−332.

Manning, B. D., & Cantley, L. C. (2003). Rheb fills a GAP between TSC and TOR. *Trends Biochem Sci 28*, 573−576.

Marcus, D. L., Strafaci, J. A., Miller, D. C., Masia, S., Thomas, C. G., Rosman, J., et al. (1998). Quantitative neuronal c-fos and c-jun expression in Alzheimer's disease. *Neurobiol Aging 19*, 393−400.

Maroney, A. C., Glicksman, M. A., Basma, A. N., Walton, K. M., Knight, E. J., Murphy, C. A., et al. (1998). Motoneuron apoptosis is blocked by CEP-1347 (KT 7515), a novel inhibitor of the JNK signaling pathway. *J Neurosci 18*, 104−111.

Maroney, A. C., Finn, J. P., Bozyczko-Coyne, D., O'Kane, T. M., Neff, N. T., Tolkovsky, A. M., et al. (1999). CEP-1347 (KT7515), an inhibitor of JNK activation, rescues sympathetic neurons and neuronally differentiated PC12 cells from death evoked by three distinct insults. *J Neurochem 73*, 1901−1912.

Maroney, A. C., Finn, J. P., Connors, T. J., Durkin, J. T., Angeles, T., Gessner, G., et al. (2001). Cep-1347 (KT7515), a semisynthetic inhibitor of the mixed lineage kinase family. *J Biol Chem 276*, 25302−25308.

Marsden, C. D. (1990). Parkinson's disease. *Lancet 335*, 948−952.

Martin, D. P., Schmidt, R. E., DiStefano, P., Lowry, O., Carter, J., & Johnson, E. (1988). Inhibitors of protein synthesis and RNA synthesis prevent neuronal death caused by nerve growth factor deprivation. *J Cell Biol 106*, 829−844.

Mata, I. F., Wedemeyer, W. J., Farrer, M. J., Taylor, J. P., & Gallo, K. A. (2006). LRRK2 in Parkinson's disease: protein domains and functional insights. *Trends Neurosci 29*, 286−293.

Mathiasen, J. R., McKenna, B. A., Saporito, M. S., Ghadge, G. D., Roos, R. P., Holskin, B. P., et al. (2004). Inhibition of mixed lineage kinase 3 attenuates MPP(+)-induced neurotoxicity in SH-SY5Y cells. *Brain Res 1003*, 86−97.

Maundrell, K., Antonsson, B., Magnenat, E., Camps, M., Muda, M., Chabert, C., et al. (1997). Bcl-2 undergoes phosphorylation by c-Jun N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J Biol Chem 272*, 25238−25242.

Mayeux, R. (2003). Epidemiology of neurodegeneration. *Annu Rev Neurosci 26*, 81−104.

McCormick, F. (2004). Cancer: survival pathways meet their end. *Nature 428*, 267−269.

Mesner, P. W., Winters, T. R., & Green, S. H. (1992). Nerve growth factor withdrawal-induced cell death in neuronal PC12 cells resembles that in sympathetic neurons. *J Cell Biol 119*, 1669−1680.

Messina, A., Jaworowski, A., & Bell, C. (1996). Detection of jun but not fos protein during developmental cell death in sympathetic neurons. *J Comp Neurol 372*, 544−550.

Moore, D. J., West, A. B., Dawson, V. L., & Dawson, T. M. (2005). Molecular pathophysiology of Parkinson's disease. *Annu Rev Neurosci 28*, 57−87.

Mota, M., Reeder, M., Chernoff, J., & Bazenet, C. E. (2001). Evidence for a role of mixed lineage kinases in neuronal apoptosis. *J Neurosci 21*, 4949−4957.

Murakata, C., Kaneko, M., Gessner, G., Angeles, T. S., Ator, M. A., O'Kane, T. M., et al. (2002). Mixed lineage kinase activity of indolocarbazole analogues. *Bioorg Med Chem Lett 12*, 147−150.

Musti, A. M., Treier, M., & Bohmann, D. (1997). Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science 275*, 400−402.

Namikawa, K., Honma, M., Abe, K., Takeda, M., Mansur, K., Obata, T., et al. (2000). Akt/protein kinase B prevents injury-induced motoneuron death and accelerates axonal regeneration. *J Neurosci 20*, 2875−2886.

Oo, T. F., Henchcliffe, C., James, D., & Burke, R. E. (1999). Expression of c-fos, c-jun, and c-jun N-terminal kinase (JNK) in a developmental model of induced apoptotic death in neurons of the substantia nigra. *J Neurochem 72*, 557−564.

Oppenheim, R. W. (1991). Cell death during development of the nervous system. *Annu Rev Neurosci 14*, 453−501.

Ozelius, L. J., Senthil, G., Saunders-Pullman, R., Ohmann, E., Deligtisch, A., Tagliati, M., et al. (2006). LRRK2 G2019S as a cause of Parkinson's disease in Ashkenazi Jews. *N Engl J Med 354*, 424−425.

Paisan-Ruiz, C., Jain, S., Evans, E. W., Gilks, W. P., Simon, J., van der, B. M., et al. (2004). Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. *Neuron 44*, 595−600.

Park, H. S., Kim, M. S., Huh, S. H., Park, J., Chung, J., Kang, S. S., et al. (2002). Akt (protein kinase B) negatively regulates SEK1 by means of protein phosphorylation. *J Biol Chem 277*, 2573−2578.

Parkinson Study Group. (2004). The safety and tolerability of a mixed lineage kinase inhibitor (CEP-1347) in PD. *Neurology 62*, 330−332.

Pirvola, U., Xing-Qun, L., Virkkala, J., Saarma, M., Murakata, C., Camoratto, A. M., et al. (2000). Rescue of hearing, auditory hair cells, and neurons by CEP-1347/KT7515, an inhibitor of c-Jun N-terminal kinase activation. *J Neurosci 20*, 43−50 (2000).

Raff, M. C., Whitmore, A. V., & Finn, J. T. (2002). Axonal self-destruction and neurodegeneration. *Science 296*, 868−871.

Ries, V., Henchcliffe, C., Kareva, T., Rzhetskaya, M., Bland, R. J., During, M. J., et al. (2006). Oncoprotein Akt/PKB: trophic effects in murine models of Parkinson's Disease. *Proc Natl Acad Sci U S A 103*, 18757−18762.

Saporito, M. S., Brown, E. R., Carswell, S., DiCamillo, A. M., Miller, M. S., Murakata, C., et al. (1998). Preservation of cholinergic activity and prevention of neuron death by CEP-1347/KT-7515 following excitotoxic injury of the nucleus basalis magnocellularis. *Neuroscience 86*, 461−472.

Saporito, M. S., Brown, E. M., Miller, M. S., & Carswell, S. (1999). CEP-1347/KT-7515, an inhibitor of c-jun N-terminal kinase activation, attenuates the 1-methyl-4-phenyl tetrahydropyridine-mediated loss of nigrostriatal dopaminergic neurons In vivo. *J Pharmacol Exp Ther 288*, 421−427.

Saporito, M. S., Thomas, B. A., & Scott, R. W. (2000). MPTP activates c-Jun NH(2)-terminal kinase (JNK) and its upstream regulatory kinase MKK4 in nigrostriatal neurons in vivo. *J Neurochem 75*, 1200−1208.

Saporito, M. S., Hudkins, R. L., & Maroney, A. C. (2002). Discovery of CEP-1347/KT-7515, an inhibitor of the JNK/SAPK pathway for the treatment of neurodegenerative diseases. *Prog Med Chem 40*, 23−62.

Sarbassov, D. D., Guertin, D. A., Ali, S. M., & Sabatini, D. M. (2005). Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science 307*, 1098−1101.

Sauer, H., & Oertel, W. H. (1994). Progressive degeneration of nigrostriatal dopamine neurons following intrastriatal terminal lesions with 6 hydroxydopamine a combined retrograde tracing and immunocytochemical study in the rat. *Neuroscience 59*, 401−415.

Schmelzle, T., & Hall, M. N. (2000). TOR, a central controller of cell growth. *Cell 103*, 253−262.

Silva, R. M., Ries, V., Oo, T. F., Yarygina, O., Jackson-Lewis, V., Ryu, E. J., et al. (2005). CHOP/GADD153 is a mediator of apoptotic death in substantia nigra dopamine neurons in an in vivo neurotoxin model of parkinsonism. *J Neurochem 95*, 974−986.

Smith, W. W., Pei, Z., Jiang, H., Moore, D. J., Liang, Y., West, A. B., et al. (2005). Leucine-rich repeat kinase 2 (LRRK2) interacts with parkin, and mutant LRRK2 induces neuronal degeneration. *Proc Natl Acad Sci U S A 102*, 18676−18681.

Smith, W. W., Pei, Z., Jiang, H., Dawson, V. L., Dawson, T. M., & Ross, C. A. (2006). Kinase activity of mutant LRRK2 mediates neuronal toxicity. *Nat Neurosci 9*, 1231−1233.

Staal, S. P., Hartley, J. W., & Rowe, W. P. (1977). Isolation of transforming murine leukemia viruses from mice with a high incidence of spontaneous lymphoma. *Proc Natl Acad Sci U S A 74*, 3065−3067.

Tapon, N., Nagata, K., Lamarche, N., & Hall, A. (1998). A new rac target POSH is an SH3-containing scaffold protein involved in the JNK and NF-kappaB signalling pathways. *EMBO J 17*, 1395−1404.

Tatton, N. A., & Kish, S. J. (1997). In situ detection of apoptotic nuclei in the substantia nigra compacta of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice using terminal deoxynucleotidyl transferase labeling and acridine orange. *Neuroscience 77*, 1037−1048.

Teismann, P., Tieu, K., Choi, D. K., Wu, D. C., Naini, A., Hunot, S., et al. (2003). Cyclooxygenase-2 is instrumental in Parkinson's disease neurodegeneration. *Proc Natl Acad Sci U S A 100*, 5473−5478.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science 267*, 1456−1462.

Tournier, C., Dong, C., Turner, T. K., Jones, S. N., Flavell, R. A., & Davis, R. J. (2001). MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes Dev 15*, 1419−1426.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., et al. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. *Science 287*, 664−666.

van der Heide, L. P., Ramakers, G. M., & Smidt, M. P. (2006). Insulin signaling in the central nervous system: learning to survive. *Prog Neurobiol 79*, 205−221.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat Rev Cancer 2*, 489−501.

Waldmeier, P., Bozyczko-Coyne, D., Williams, M., & Vaught, J. L. (2006). Recent clinical failures in Parkinson's disease with apoptosis inhibitors underline the need for a paradigm shift in drug discovery for neurodegenerative diseases. *Biochem Pharmacol*.

Watson, A., Eilers, A., Lallemand, D., Kyriakis, J., Rubin, L. L., & Ham, J. (1998). Phosphorylation of c-Jun is necessary for apoptosis induced by survival signal withdrawal in cerebellar granule neurons. *J Neurosci 18*, 751−762.

Wendel, H. G., De Stanchina, E., Fridman, J. S., Malina, A., Ray, S., Kogan, S., et al. (2004). Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. *Nature 428*, 332−337.

Wessel, T. C., Joh, T. H., & Volpe, B. T. (1991). In situ hybridization analysis of c-fos and c-jun expression in the rat brain following transient forebrain ischemia. *Brain Res 567*, 231−240.

West, A. B., Moore, D. J., Biskup, S., Bugayenko, A., Smith, W. W., Ross, C. A., et al. (2005). Parkinson's disease-associated mutations in leucine-rich repeat kinase 2 augment kinase activity. *Proc Natl Acad Sci U S A 102*, 16842−16847.

Whitmarsh, A. J., Kuan, C. Y., Kennedy, N. J., Kelkar, N., Haydar, T. F., Mordes, J. P., et al. (2001). Requirement of the JIP1 scaffold protein for stress-induced JNK activation. *Genes Dev 15*, 2421−2432.

Xia, X. G., Harding, T., Weller, M., Bieneman, A., Uney, J. B., & Schulz, J. B. (2001). Gene transfer of the JNK interacting protein-1 protects dopaminergic neurons in the MPTP model of Parkinson's disease. *Proc Natl Acad Sci U S A 98*, 10433−10438.

Xia, Z., Dickens, M., Raingeaud, J., Davis, R. J., & Greenberg, M. E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science 270*, 1326−1331.

Xu, Z., Maroney, A. C., Dobrzanski, P., Kukekov, N. V., & Greene, L. A. (2001). The MLK family mediates c-Jun N-terminal kinase activation in neuronal apoptosis. *Mol Cell Biol 21*, 4713−4724.

Xu, Z., Kukekov, N. V., & Greene, L. A. (2003). POSH acts as a scaffold for a multiprotein complex that mediates JNK activation in apoptosis. *EMBO J 22*, 252−261.

Yamamoto, K., Ichijo, H., & Korsmeyer, S. J. (1999). BCL-2 is phosphorylated and inactivated by an ASK1/Jun N-terminal protein kinase pathway normally activated at G(2)/M. *Mol Cell Biol 19*, 8469−8478.

Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., et al. (1997). Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature 389*, 865−870.

Yao, R., & Cooper, G. M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. *Science 267*, 2003−2006.

Zhou, H., Li, X. M., Meinkoth, J., & Pittman, R. N. (2000). Akt regulates cell survival and apoptosis at a postmitochondrial level. *J Cell Biol 151*, 483−494.

Zigmond, M. J., Abercrombie, E. D., Berger, T. W., Grace, A. A., & Stricker, E. M. (1990). Compensations after lesions of central dopaminergic neurons: some clinical and basic implications. *Trends Neurosci 13*, 290−296.

Zimprich, A., Biskup, S., Leitner, P., Lichtner, P., Farrer, M., Lincoln, S., et al. (2004). Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. *Neuron 44*, 601−607.