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14. ABSTRACT
A longstanding concept in neuroscience has been that the mature mammalian brain is incapable of axon regeneration. However, we have shown that it is possible to achieve long range axon growth by re-activation of intrinsic genetic programs that are active during development. We have found that activation of Akt/mTor signaling by AAV-mediated transduction with either the kinase Akt or the GTPase Rheb in a model of retrograde axonal degeneration induces axon growth by dopamine neurons. However, these molecules cannot be directly used in therapeutics because they are oncogenes. The goal of this proposal therefore is to develop a strategy to circumvent this problem. We have hypothesized that mediators downstream of mTor may diverge in their effects, making it possible to achieve axon growth without oncogenic risk. In Year 01 we had shown that one mTor target, p70S6K, is able to induce new axon growth. In Year 02 we have assessed the ability of a second mTor target, eIF4E, to induce new axon growth. We have found that transduction of dopamine neurons of the SN by AAV eIF4E at three weeks after axonal destruction with the neurotoxin 6OHDA does not induce new axon growth. This conclusion was reached by assessments based on three techniques: immunostaining for tyrosine hydroxylase-positive axons in the medial forebrain bundle (MFB); quantification by confocal optical dissection of either GFP-positive axons in the MFB in transgenic THGFP mice or of Tomato-positive axons following transduction with anterograde tracer Tomato-Tau. As anticipated, based on anatomical evidence showing an inability of AAV eIF4E to re-establish anatomical contact, we found no reversal of a toxininduced behavioral deficit. We conclude that of the two mTor targets, p70S6K and eIF4E, only p70S6K is an effective mediator of new axon growth. It will therefore be the focus of a refined, axon-targeting strategy in Year 03 of this proposal.

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INTRODUCTION

A longstanding concept in neuroscience has been that the mature mammalian central nervous system (CNS), unlike the peripheral nervous system (PNS), is incapable of axon regeneration. There are currently two principal concepts that form the basis of our understanding of the inability of the mature brain to regenerate axons. The first, and predominant, concept is that following injury to the CNS, extrinsic factors prevent axon growth (1, 2). These extrinsic factors are principally of two types: glial scar and myelin breakdown products. The second concept to account for axon regeneration failure is that as the brain matures, the intrinsic developmental genetic programs that mediate axon growth are silenced. In recent years there has been growing interest in the role played by silencing in the adult brain of these intrinsic programs. The therapeutic promise offered by the concept of silencing of axon growth programs is that it suggests that if we are able to re-activate them, it may be possible to achieve long range restorative axon growth. Such a possibility has received support from our recent studies of the activation of Akt/mTor signaling in models of retrograde axonal degeneration induced by the dopaminergic neurotoxin 6-hydroxydpamine (6OHDA). In this model, 6OHDA is injected unilaterally into the striatum of mice and within one week it induces over 80% destruction of the dopaminergic nigro-striatal projection (3). This model has been used for many years to simulate the principal neurodegeneration that occurs in human Parkinson's disease (PD). In order to accurately simulate the clinical presentation of the disease, we waited until three weeks after the lesion, when most axons have been destroyed and about 50% of neurons still survive, and then transduced the surviving dopamine neurons by use of an AAV1 vector with either a constitutively active mutant of the Akt kinase (myristoylated-Akt (MYR-Akt)) (4) or hRheb(S16H), a constitutively active mutant of the Rheb GTPase (5, 6). Rheb GTPase is activated by Akt and it is a direct activator of the mTor kinase (7) (FIGURE 1). We have observed that transduction of SN dopamine neurons with either AAV MYR-Akt or AAV hRheb(S16H) induces a remarkable reinnervation of the striatum (FIGURE 2) (5, 6). While these observations with AAV MYR-Akt and AAV hRheb(S16H) offer a promising proof-of-concept, they cannot be directly implemented as a gene therapy for humans because both of these molecules are potent oncogenes. The challenge, therefore, is to try to exploit the beneficial axon growth phenotype offered by this approach while eliminating the undesirable oncogenic phenotype. In this proposal, we seek to address this challenge by attempting to identify the critical mediators of the axon growth phenotype downstream to mTor. We postulate that at some point downstream, the paths mediating the two phenotypes must diverge. Based on our results with hRheb(S16H) we know that activation of mTor is sufficient for axon growth. mTor has two principal substrates: eukaryotic translation initiation factor 4E binding protein (4E-BP1) and p70S6K (FIGURE 1). Of the

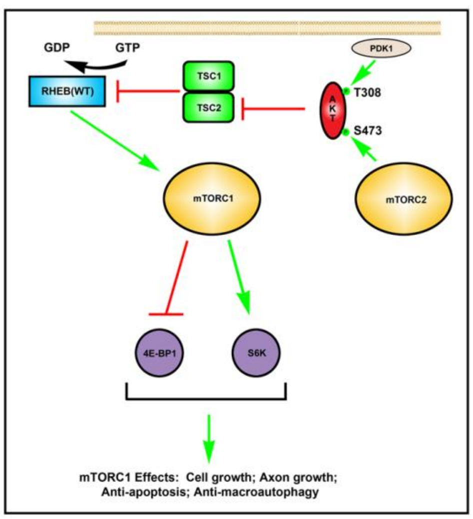

FIGURE 1. Schematic representation of Akt/Rheb/mTor signaling pathways. Following activation at the plasma membrane by phosphorylation by PDK1 and mTORC2, Akt phosphorylates and thereby inhibits the GTPase activity of the tuberous sclerosis complex (TSC). This inhibition allows accumulation of activated GTP-bound Rheb, which is a principal activator of the mTORC1 kinase. Two principal downstream substrates of mTORC1 are 4E-BP1 and p70S6K. Their phosphorylation mediates effects of mTORC1 activation.

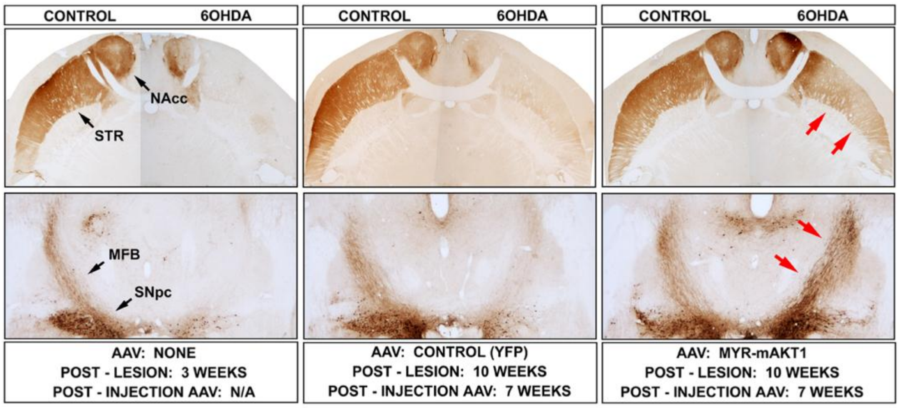

FIGURE 2. Constitutively active Akt (Myr-Akt) induces re-population of the MFB by dopaminergic axons. Horizontal sections of mouse brain, stained for TH, demonstrate restoration of dopaminergic axons within the MFB by Myr-Akt. At 3 weeks post-lesion, there are a few remaining dopaminergic neurons in the SNpc, but in this low magnification micrograph there is no remaining axonal immunostaining in the MFB or striatum (STR). Following intra-nigral administration of AAVs, very little TH immunostaining is observed in both MFB and striatum at 10 weeks post-lesion of mice given AAV YFP as control. However, robust axonal immunostaining is observed in the MFB (red arrows, bottom right panel) and in the striatum (red arrows, top right panel) of mice given AAV Myr-Akt.

two, we hypothesize that p70S6K is more likely to play a role in axon growth. P70S6K has been identified as both necessary and sufficient for axon specification in primary neurons (8). Transduction of neurons with a constitutively active form of p70S6K induced the formation of multiple axons, whereas increased expression of eIF-4E did not (8) . Although our principal hypothesis favors p70S6K in the induction of axon growth, we intend in this proposal to cover both possibilities by investigating the alternate mTor mediator, 4E-BP1, as well. The aim of this proposal therefore is to test the hypothesis that p70S6K or 4E-BP1 has the ability to induce axon regeneration in lesioned dopamine neurons. This proposal has direct therapeutic implications for the treatment of PD and other chronic neurologic diseases characterized by axon degeneration.

BODY

TASK 1 YEAR 01: To determine whether p70S6K or a constitutively active mutant, or both, are mediators of axon growth in the dopaminergic nigro-striatal projection.

In Year 01 we successfully completed this Task, and found that a constitutively active form of p70S6K can mediate new axon growth. We first created the AAV vectors for p70S6K(WT) and the constitutively active form p70S6K(del C/T389E) (9, 10). In these Experiments a 6OHDA lesion was performed, followed in three weeks by intra-nigral injection of the AAV vectors. The effects on axon growth were assessed at 12 weeks post AAV. We determined by three measures that p70S6K(del C/T389E) induced new axon growth following their destruction by neurotoxin. We determined that p70S6K(del C/T389E) partially restores axon numbers in the medial forebrain bundle (MFB), identified as TH-positive axons in the MFB. In addition, we used two methods to rule out the possibility that p70S6K(del C/T389E) was just upregulating TH expression and not inducing new axon growth. In the first, we used transgenic mice (TH-GFP) that express GFP under the TH promoter. GFP is a stable protein and not subject to endogenous regulators of TH. Second we have used an anterograde tracer technique in which the axon-targeted fusion protein Tomato-Tau is delivered to SN neurons by AAV and expression is driven by the robust chicken-beta actin promoter. By both of these methods there was a clear induction of axon growth by AAV p70S6K(del C/T389E). Based on these results we concluded that our hypotheses was confirmed, that p70S6K, a downstream target of mTORC1, is capable of recapitulating the axon growth effects of MYR-Akt and hRheb(S16H).

TASK 2. YEAR 02. To determine whether 4E-BP1, as the second principal substrate of mTORC1 signaling, is a mediator of axon growth in the nigro-striatal dopaminergic projection.

In Year 02 we successfully created the AAV vector for eIF4E, a protein translation factor that is constitutively inhibited by 4E-BP1. Experiments were performed as described for *TASK 1*. eIF4E did not induce a significant effect on striatal innervation, the number of dopaminergic axons within the medial forebrain bundle (MFB) by tyrosine hydroxylase (TH) immunohistochemistry, or the number of GFP-positive axons or Tomato-Tau-positive axons in the MFB. Given the lack of an effect of eIF4E on axon growth by these anatomical measures, we anticipated that there would not be evidence for restoration of behavioral deficits and that proved to be the case. Thus, unlike p70s6K, eIF4E did not have an ability to induce axon regrowth.

With these results for AAV eIF4E completed in Year 02 we came to an important juncture in our studies. We had originally proposed in our Statement of Work that we would devote our first two TASKs in Years 01 and 02 to a determination of which downstream substrate of the kinase mTor is responsible for its axon growth-mediating effects. After these first two years of work, and completion of TASKS 1 and 2 we have a clear and unequivocal answer: p70S6K mediates axon growth and eIF4E does not (FIGURE 3). We have therefore proceeded in *Task 3* to further refine the use of p70S6K by genetically engineering it to be targeted to axons. We hypothesize that its axon growth effects are mediated locally within the axon. If our hypothesis is correct, then the axonally targeted forms will be more potent and it will have diminished risk of off-target effects, such as oncogenesis.

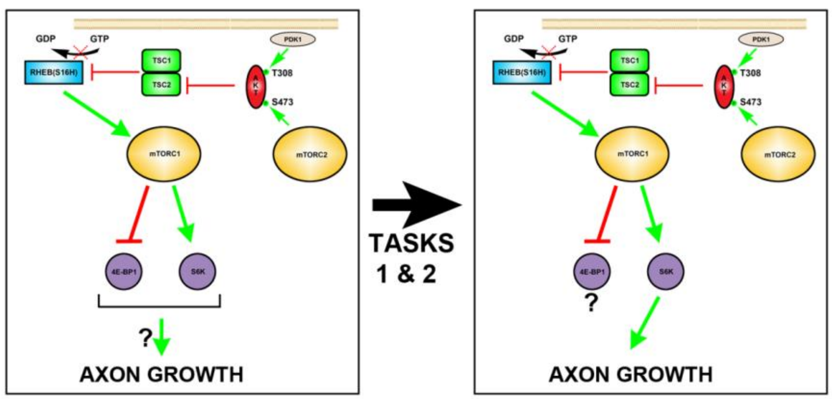

FIGURE 3. Schematic representation of the new knowledge obtained by completion of TASKS 1 and 2. We have previously shown that the kinase Akt (symbolized by the vertical red ellipse) can induce robust re-growth of dopaminergic axons in the adult brain weeks following their destruction by 6OHDA (Kim et al, *Ann Neurol*, 2011). This effect is mediated through mTor signaling because it can be entirely recapitulated by the principal mTor activator, the Rheb GTPase (blue rectangle), demonstrated by transduction with a constitutively active form (hRheb(S16H)). This mutant is resistant to GTPase activation (symbolized by the red cross), and thereby the inhibition induced by the tuberous sclerosis complex (green rectangles) (Kim et al, *Mol Therapy*, 2012). In order to refine gene therapies capable of inducing axon growth by minimizing the potential off-target effects of either Akt or Rheb, we proposed in our Statement of Work to determine which of the principal downstream signaling pathways of mTor1, the regulation of eukaryotic initiation factor 4E by 4E-BP1, or the activation of the p70 S6 kinase (or both), mediates axon growth. Our completion of TASKs 1 and 2 has clearly shown that the axon re-growth induced by activation of mTor1 is mediated by the p70S6 kinase. Now that we have identified p70S6K as the mediator of axon re-growth by these signaling pathways, we will now proceed with TASK 3 and attempt to further refine its use in gene therapy for axon restoration. We propose to determine whether p70S6K acts locally within the axon to mediate its effects. We will achieve this goal by the evaluation of axonally-targeted forms. If this approach is successful, it will further minimize off-target effects and it will enhance the potency of p70S6K as an axon growth mediator.

TASK 3. YEARS 03-04. To determine whether an axon targeting strategy affects the ability of p70S6K to induce axon growth.

In Year 03, we have created the following axon-targeting vectors for p70S6K(del C/T389E), the constitutively active form of p70S6K:

AAV cAPP-pS6K(CA): (CA: constitutively active). This construct is targeted to axons by 15 C-terminus amino acids of amyloid precursor protein (cAPP). cAPP was found in our recent publication in *Gene Therapy* (2013) to be the most effective known axon-targeting motif. In that study the motif was placed at the N-terminus as it had been in previously published studies.

AAV P70SK6(CA)-cAPP: This construct again uses the cAPP axon-targeting motif, but places it at the C-terminus as it is in its native location in the APP protein.

AAV cAPP-p70S6K(CA)-TAU-ZIP: This construct supplements the peptide-based cAPP axon targeting motif with an mRNA-based motif, the 3'UTR axon-targeting 'zip code' of the microtubule-binding protein tau. In our *Gene Therapy* publication, it was the most potent mRNA-based motif.

AAV 3XNLS-pS6K(CA): This construct includes a nuclear localization signal (NLS) and it will target the constitutively active form of p70S6K away from the axon, into the nucleus.

AAV P70SK6(CA)-CaMKII-3'UTR. This construct targets mRNA away from the axon, towards the dendrites.

AAV p70S6K(del C/T389E), the constitutively active form of p70S6K, without axonal targeting, will serve as a comparison control for the targeted forms.

However, we encountered an issue for the three axonal targeted constructs. Although we have routinely detected the FLAG epitope in numerous constructs in our prior work, we were unable to detect FLAG in these constructs. This problem made it impossible to determine transduction efficiency, and, most importantly, to determine relative ability to achieve axon targeting. Fortunately, we were able to establish a 'work around' for this problem; we established an immunostaining protocol for p70S6K itself.

With the new immunostaining protocol for p70S6K we made the novel observation that endogenous p70S6K is abundantly expressed in axons of the medial forebrain bundle. This observation is of interest because it suggests that this protein plays a normal physiologic role in the maintenance of these axons. If such is the case, then it would be an especially good candidate for restoration of axons in this system. Given that endogenous p70S6K is expressed in axons, we were not surprised to observe that the transgene p70S6K(CA), without an axon targeting sequence, was also expressed in axons. Disappointingly, however, the abundance of p70S6K(CA) protein expression in axons was not increased by any of the axon targeting motifs. The reason for this may be that since endogenous p70S6K is normally trafficked to axons, the mechanisms that mediate this localization may have achieved maximal axonal expression for the transgene p70S6K(CA), such that there is no further enhancement by the addition other axon targeting motifs. In view of this unexpected result, we have modified our strategy to evaluate the role of normal axonal expression on the ability of p70S6K to mediate axon growth. If the maximum expression in axons is achieved by non-targeted p70S6K(CA), then we can assess the importance of axon expression by performing the converse experiment and divert this molecule away from the axon compartment. This will be done by use of a nuclear targeting signal, to traffic to the nucleus, and by the use of a CaMKII 3'UTR motif that traffics mRNAs to dendrites. We have previously achieved dendritic targeting with this motif (Padmanabhan et al, *Gene Therapy*, 2014).

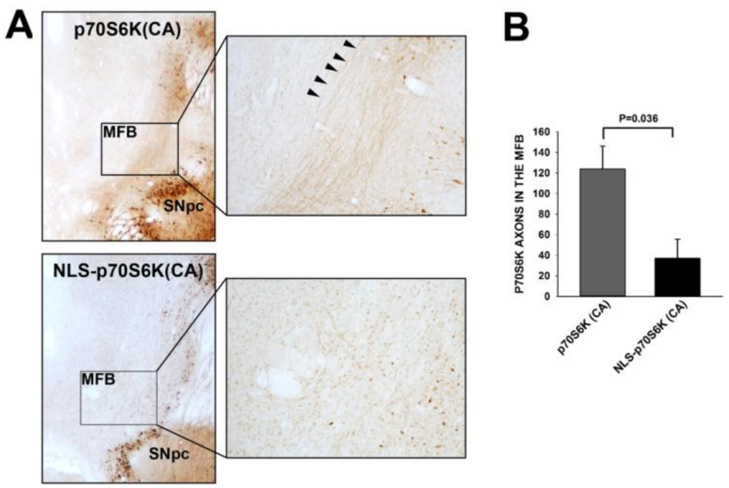

FIGURE 4. The incorporation of a nuclear localization signal (NLS) in p70S6K(CA) diminishes its expression in axons. **A.** In the upper panels, immunostaining for p70S6K demonstrates axons in the medial forebrain bundle (MFB). The left panel, at low power, demonstrates the MFB adjacent to the SNpc. The right panel, at higher power, demonstrates individual axons in the MFB (one is marked with arrowheads). The lower panels show that when an NLS is incorporated into the p70S6K(CA) sequence, the MFB has fewer axons. **B.** This difference is shown quantitatively. P70S6K-positive axons In the MFB were counted using a stereologic technique. There were fewer axons in the MFB following incorporation of the NLS (CA: 123.9 ± 22.2 (SEM); NLS: 37.3 ± 18.8, p = 0.036, t-test).

We have completed two accomplishments to pursue this strategy: (1) We have performed experimental analysis of axon labeling by non-targeted p70S6K(CA) in comparison to AAV 2/1 3X NLS p70S6K(CA). The NLS signal is clearly effective in diverting p70S6K away from axons (see FIGURE 4). (2) We have produced the AAV 2/1 p70S6K(CA) CaMKII 3'UTR-ZIP, and the construct is now undergoing characterization for toxicity and transduction efficiency.

KEY RESEARCH ACCOMPLISHMENTS

- AAV2/1 vectors have been created for p70S6K(WT), p70S6K(del C/T389E), and eIF4E.
- AAV p70S6K(del C/T389E) has been shown to induce new axon growth in SN dopamine neurons following axon destruction, thus recapitulating the abilities of MYR-Akt and hRheb(S16H) to do so.
- The new axon growth induced by p70S6K(del C/T389E) is functional; it is able to restore behavioral deficits induced by 6OHDA lesion.
- AAV eIF4E does not recapitulate the axon growth effects of MYR-Akt and hRheb(S16H).
- AAV2/1 vectors have been created to target p70S6K(del C/T389E) to axons: AAV cAPP-pS6K(CA), AAV P70SK6(CA)-cAPP, and AAV cAPP-p70S6K(CA)-TAU-ZIP.
- We have established an immunohistochemical protocol for staining p70S6K and have demonstrated that both endogenous p70S6K and our non-targeted transgene, p70S6K(del C/T389E) are expressed in axons.
- We have found that diverse approaches to axon targeting do not achieve a higher level of axonal expression than observed with endogenous p70S6K or our non-targeted transgene, p70S6K(del C/T389E).
- This observation has led us to change our strategy; we will now create mutant forms of p70S6K(del C/T389E) that are targeted away from the axon by either a nuclear localization signal or by a dendrite targeting signal.

REPORTABLE OUTCOMES:

Abstracts

Padmanabhan S, Kareva T, Kholodilov N, Burke RE. Gene therapy for axon protection and restoration in Parkinson's disease: Comparison of axon targeting strategies. Society for Neuroscience, 2011.

Padmanabhan S, Yarygina O, Kareva T, Kholodilov N, Burke RE. A constitutively active form of p70S6K induces axon growth in the nigrostriatal dopaminergic system. Society for Neuroscience, 2012.

Padmanabhan S, Kareva T, Al-Ribadi D, Kholodilov N, Burke RE. Ribosomal protein S6 kinase (p70S6K) is a mediator of Akt/Rheb/mTOR-induced axon regeneration following injury in the adult brain. Meeting on Axon Guidance, Synapse Formation & Regeneration, Cold Spring Harbor, NY, 2014.

Padmanabhan S, Kareva T, Yarygina O, Oo TF, Kholodilov N, Burke RE. Evaluation of a possible role for the GTPase Rap1B in the induction of new axon growth in the adult nigrostriatal dopaminergic system. Society for Neuroscience, 2014.

Publications

Padmanabahn S, Kareva T, Kholodilov N, Burke RE. Quantitative morphological comparison of axon targeting strategies for gene therapies directed to the nigro-striatal projection. Gene Therapy, 2014, 21:115-122.

CONCLUSIONS

Based on our results in Years 01 & 02, we conclude that our fundamental strategy, to attempt to recapitulate the axon growth effects of MYR-AKT and hRheb(S16H) with mediators downstream of mTORC1 is successful, because we have achieved axon growth with a constitutively active form of p70S6K. In Year 02 we determined that the other principal mTor target, eIF4E, does not have axon growth induction capacity. In Year 03 we have learned that endogenous p70S6k and our constitutively active transgene, p70S6K(del C/T389E), are both expressed in axons. We found that diverse approaches to axon targeting were unable to augment the normal axon targeting of these molecules. In order to determine the importance of expression in axons to the ability of p70S6K(del C/T389E) to induce new axon growth, we will now use a converse strategy and divert it away from axons. This will be done using a nuclear localization signal and a dendrite targeting signal. In Year 03 we have demonstrated that the NLS does indeed diminish p70S6K(del C/T389E) expression in axons. The evaluation of the dendritic targeting signal, CaMKII-3'UTR, is underway.

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