Regulation of axon growth by the JIP1–AKT axis

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ABSTRACT

The polarisation of developing neurons to form axons and dendrites is required for the establishment of neuronal connections leading to proper brain function. The protein kinase AKT and the MAP kinase scaffold protein JNK-interacting protein-1 (JIP1) are important regulators of axon formation. Here we report that JIP1 and AKT colocalise in axonal growth cones of cortical neurons and collaborate to promote axon growth. The loss of AKT protein from the growth cone results in the degradation of JIP1 by the proteasome, and the loss of JIP1 promotes a similar fate for AKT. Reduced protein levels of both JIP1 and AKT in the growth cone can be induced by glutamate and this coincides with reduced axon growth, which can be rescued by a stabilized mutant of JIP1 that rescues AKT protein levels. Taken together, our data reveal a collaborative relationship between JIP1 and AKT that is required for axon growth and can be regulated by changes in neuronal activity.

KEY WORDS: Axon, JIP1, AKT, Proteasome

INTRODUCTION

As neurons develop in the brain, they polarise resulting in a single axon and multiple dendrites. The axon conducts information while the cell body and dendrites integrate this information by making multiple synaptic connections. The polarisation of neurons is therefore essential for the formation of neuronal networks and the function of the brain (Barnes and Polleux, 2009). A number of factors determining axon formation and growth have been uncovered, including the localisation of the centrosome and Golgi, the regulation of gene expression, and alterations in cytoskeletal dynamics mediated by many signalling proteins. These include GTPases of the Ras and Rho families, Par complexes, ubiquitin ligase complexes that target key proteins for degradation, and the LKB1 and phosphoinositide 3-kinase (PI3K) complexes, ubiquitin ligase complexes that target key proteins for degradation, and the LKB1 and phosphoinositide 3-kinase (PI3K) pathways (Barnes and Polleux, 2009; Cheng and Poo, 2012; de la Torre-Ubieta and Bonni, 2011).

The protein kinase AKT is a component of the PI3K pathway. It is recruited to the PI3K activity-dependent phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)P3] in the membrane where it is activated by 3-phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylation at Thr308 (Bhaskar and Hay, 2007). A second phosphorylation event at Ser473, which can be mediated by the mTOR-containing complex TORC2, is required for full AKT activity and may also regulate substrate selection (Bhaskar and Hay, 2007). In developing neurons, AKT preferentially localises to newly forming axons rather than dendrites (Shi et al., 2003) and it has been proposed that this differential localisation of AKT is maintained by its proteasomal degradation in the dendrites (Yan et al., 2006). Indeed, inhibition of the proteasome leads to a loss of polarisation and multiple AKT-positive neurites (Yan et al., 2006). The presence of activated AKT is crucial for axon formation through its targeting of effector proteins that regulate the cytoskeleton and promote axon extension, in particular through GSK3β–CRMP2 (Jiang et al., 2005; Yoshimura et al., 2005).

It has previously been reported that AKT associates with the JNK MAP kinase scaffold protein JIP1 (Kim et al., 2002; Song and Lee, 2005a). JIP1 can also bind to multiple components of the JNK pathway and facilitate JNK activation (Whitmarsh et al., 1998). In response to excitotoxicity or oxygen–glucose deprivation, JIP1 mediates JNK-dependent apoptosis of hippocampal neurons (Whitmarsh et al., 2001). It has been shown that AKT dissociates from JIP1 in response to excitotoxic or metabolic stress thereby allowing JIP1 to recruit JNK and enhance its activation (Kim et al., 2002; Song and Lee, 2005a; Song and Lee, 2005b). Work in our laboratory has demonstrated that JIP1, in addition to its role in stress responses, is required for the polarisation of cultured cortical neurons and for axon growth (Dajas-Bailador et al., 2008). JIP1 specifically localises to the growth cone of the developing axon through an interaction with the motor protein kinesin-1 (Verhey et al., 2001; Dajas-Bailador et al., 2008) and, in association with kinesin-1, it might regulate axonal vesicle transport through interactions with transmembrane proteins, including APP (Muresan and Muresan, 2005; Horiuchi et al., 2005; Whitmarsh, 2006; Fu and Holzbaur, 2013).

In this study we explore the relationship between JIP1 and AKT during axon outgrowth in cultured cortical neurons. We demonstrate that JIP1 and AKT colocalise in the growth cone of the developing axon and maintain each other’s stability. The loss of either protein promotes the degradation of the other by the proteasome and a cessation of axon growth. Furthermore, the levels of JIP1 and AKT in the axon growth cone are regulated by glutamate receptor stimulation.

RESULTS

JIP1 and AKT stabilize each other in axonal growth cones

JIP1 and AKT localise to axonal growth cones (Shi et al., 2003; Dajas-Bailador et al., 2008) and have been reported to form complexes (Kim et al., 2002; Song and Lee, 2005a); however, it remains unclear whether these proteins can collaborate to regulate axon formation and function. To investigate this we used cultured...
primary cortical neurons from mouse embryos. In vitro, these neurons undergo a series of developmental stages whereby they extend a number of neurites (stages 1 and 2) prior to polarisation, at which point one of the neurites forms an axon and the others develop into dendrites (stage 3) (Dotti et al., 1988). At stage 2, AKT is reported to be present at the tips of all the neurites (Yan et al., 2006) whereas JIP1 has already localised to the future axon (Dajas-Bailador et al., 2008). At stage 3, JIP1 and AKT colocalise in the growth cones of newly formed axons along with the axonal-specific microtubule binding protein Tau (Fig. 1A; supplementary material Fig. S1). To determine whether JIP1 plays a scaffold role in maintaining AKT at the axon tip, shRNA-mediated knockdown of JIP1 was performed. A substantial reduction in JIP1 protein was observed by immunoblotting (Fig. 1B). JIP1 knockdown had minimal effect on the total AKT protein level in the neurons as determined by immunoblotting (Fig. 1B); however, examination of immunostained axonal growth cones revealed a significant loss of AKT (Fig. 1C,D). There were no obvious changes in the AKT levels in the cell bodies and axon shafts (Fig. 1C), thus explaining why we do not observe a substantial reduction in total AKT protein in the neurons (Fig. 1B). To confirm that the reduced AKT protein level in the growth cone was due to the loss of JIP1 and not an off-target effect of the shRNA, we re-introduced recombinant JIP1 back into the neurons and rescued the AKT staining (Fig. 1C,D). We next determined whether the localisation of JIP1 to the axon tip was required to maintain the AKT level there. JIP1 localises to the growth cone through its interaction with the kinesin-1 motor protein (Verhey et al., 2001). The kinesin-1-binding site on JIP1 resides at its C-terminus and mutation of Tyr705 to Ala within this region prevents binding and results in the loss of JIP1 localisation to the growth cone (Verhey et al., 2001; Dajas-Bailador et al., 2008). Unlike wild-type JIP1, the expression of the JIP1 Y705A mutant did not rescue the loss of AKT protein in the growth cone following knockdown of endogenous JIP1 (Fig. 1C,D). These data suggest that JIP1 is required to maintain AKT protein levels in the axon growth cone. To further understand the relationship between JIP1 and AKT, we investigated whether the loss of AKT affected JIP1 localisation or stability. To do this we knocked down AKT protein levels with shRNAs directed against the three AKT isoforms (AKT1, AKT2 and AKT3). This led to a decrease in total JIP1 protein (Fig. 1E) and in JIP1 immunostaining of the axon tip (Fig. 1F,G). The loss of axonal JIP1 could be rescued by re-introducing recombinant human AKT1 into the neurons (Fig. 1F,G). Taken together, these data suggest that the AKT and JIP1 support each other’s presence in axonal growth cones.

AKT and JIP1 protect each other from degradation by the proteasome

One possibility was that AKT and JIP1 protect each other from proteasome-mediated degradation in the axon tip. To address this, neurons were incubated with the proteasome inhibitor MG132. This rescued the loss of JIP1 in the axon tips of AKT knockdown neurons (Fig. 2A) and also the reduction of AKT in the axon tips of JIP1 knockdown neurons (Fig. 2B). This suggests that the colocalisation of JIP1 and AKT to the axon growth cone prevents their proteasome-dependent degradation. As the AKT pathway mediates many cellular processes, including transcriptional responses, we determined whether the loss of JIP1 protein that occurs upon AKT knockdown might also reflect reduced transcription of the Jip1 gene. However, no changes in transcript levels were observed (Fig. 2C), thus providing additional evidence that the major mechanism of regulation is through protein stability.

The role of proteasomal regulation of AKT and JIP1 in axon formation

It is known that the inhibition of the proteasome prevents neuronal polarisation and results in multiple neurites with high levels of AKT at the tips (Yan et al., 2006). We confirmed the presence of AKT at many neurite tips following inhibition of the proteasome (Fig. 3A,C) and also demonstrated that JIP1 was present in many neurite tips (Fig. 3B,C). Indeed, JIP1 and AKT were colocalised in the tips (Fig. 3C). These data demonstrate that inhibition of the proteasome leads to loss of the asymmetric distribution of AKT and JIP1 to a single axon. This correlated with a significant reduction in neuronal polarisation (Fig. 3D) with the majority of the neurites positive for the axonal marker protein Tau (Fig. 3E,F). These data suggest a strong correlation between the presence of JIP1 and AKT in a neurite and it displaying axon-like properties, and support a model whereby the specific localisation of both JIP1 and AKT proteins to the axon is an important mechanism in the regulation of neuronal polarisation and axon growth.

Neuronal activity regulates JIP1 and AKT stability

In addition to the role of JIP1 and AKT in axon formation, we were interested in determining whether the JIP1–AKT axis is responsive to neuronal activity. Glutamate is the major excitatory neurotransmitter in the central nervous system and the principal neurotransmitter of cortical efferent systems (Fonnum, 1984). Previous studies have demonstrated that JIP1 can be regulated following stimulation of the N-methyl-D-aspartate (NMDA) class of glutamate receptors (Kim et al., 2002; Kennedy et al., 2007; Centeno et al., 2007). We exposed developing cultured neurons to glutamate and this led to a reduction in JIP1 protein levels that could be rescued by the NMDA receptor antagonist MK-801, but not by the AMPA/kainate antagonist CNQX (Fig. 4A). This indicated that glutamate was acting through NMDA receptors to decrease JIP1 protein levels. We next determined whether glutamate was acting locally on NMDA receptors in the axon or through receptors at the cell body or in dendrites, thereby regulating JIP1 levels from a distance. To distinguish these possibilities, we plated neurons in microfluidic chambers that allow the compartmentalisation and fluidic isolation of the axons away from the cell bodies and dendrites (Fig. 4B). Application of glutamate to the axonal compartment was sufficient to reduce JIP1 immunostaining in the axon tip, indicating that glutamate could act directly on the axon to regulate JIP1 stability (Fig. 4C,D). To determine whether JIP1 stability was sensitive to the endogenous activation of glutamate receptor neurotransmission, we exposed neurons to potassium chloride (KCl). This causes cortical neurons to undergo membrane depolarisation and subsequent activation of voltage-sensitive channels leading to glutamate release. KCl caused a reduction in overall JIP1 protein levels and this was rescued by pre-incubation of neurons with an inhibitor of the L-type voltage-dependent calcium channel (nifedipine) or the NMDA receptor antagonist MK801 (Fig. 4E). Furthermore, KCl caused a reduction of both JIP1 and AKT proteins in axon growth cones (Fig. 4F,G). The activation of NMDA receptors by glutamate increases calcium ion influx; therefore we investigated whether this contributed to the loss of JIP1 stability. EGTA was used to chelate extracellular calcium ions and prevent the NMDA-dependent calcium ion influx. This rescued the loss of JIP1 protein that occurred following glutamate treatment (Fig. 4H) and confirmed that intracellular calcium signalling is required to destabilize JIP1.
Fig. 1. JIP1 and AKT are required to maintain their respective protein levels in axonal growth cones. (A) Cortical neurons were fixed at 3 days in vitro (d.i.v.) and stained with anti-JIP1, anti-AKT and anti-Tau antibodies. Left panels: JIP1, AKT and Tau localise to the tip of the emerging axon (indicated by a white arrow). Scale bar: 40 μm. Right panels: magnification of boxed growth cone demonstrating co-localisation of JIP1, AKT and Tau in the axon tip. Scale bar: 10 μm. (B) Immunoblot of JIP1 and AKT protein levels in lysates of neurons treated with lentivirus expressing JIP1 shRNA or control shRNA. Tubulin was used to control for equal protein loading. (C) Immunostaining of AKT in axon growth cones and cell bodies of neurons electroporated with plasmids expressing JIP1 shRNA or control shRNA. AKT immunostaining was also assessed in neurons transfected with pCDNA3-T7-JIP1 or pCDNA3-T7-JIP1(Y705A). The latter plasmid expresses a JIP1 mutant that does not bind kinesin-1. Scale bars: 10 μm. (D) Quantification of AKT immunostaining in axon tips after electroporation of neurons with plasmids expressing control shRNA or JIP1 shRNA. Neurons expressing JIP1 shRNA were also transfected with either pCMV5 or pCMV5-HA-AKT1. Scale bar: 10 μm. (E) Neurons were subjected to lentiviral-mediated shRNA knockdown of AKT1/2/3 and the protein level of JIP1 was measured by immunoblotting. A representative immunoblot is shown as is the quantification of blots from three experiments. (F) Immunostaining of JIP1 in axon growth cones after shRNA knockdown of AKT1/2/3 with or without transfection of pCMV5 or pCMV5-HA-AKT1 (*P<0.05, **P<0.01).
Glutamate stimulation causes the loss of JIP1 and AKT proteins in the growth cone through the JNK pathway and the proteasome

As we had evidence that the proteasome regulates JIP1 and AKT stability during axon development (Figs 2, 3), we determined whether the loss of JIP1 and AKT in the growth cone in response to glutamate stimulation was also being mediated by the proteasome. The pre-incubation of glutamate-stimulated neurons with the proteasome inhibitor MG132 rescued both JIP1 and AKT protein levels in axon tips, indicating that activation of glutamate neurotransmission can regulate the stability of these proteins (Fig. 5A–C). It has previously been reported that activation of kainate glutamate receptors in an excitotoxic model can cause the dissociation of the JIP1–AKT complex (Kim et al., 2002). We did not observe neuronal loss in our experiments following stimulation of neurons with glutamate (100 μM, 3 hours; supplementary material Fig. S2) suggesting that the loss of JIP1 and AKT in axons is unlikely to be due to excitotoxicity. However, it raised the possibility that glutamate promotes the dissociation of the JIP1–AKT complex leading to their instability. Co-immunoprecipitation experiments demonstrated that glutamate stimulation did decrease JIP1 binding to AKT (Fig. 5D). This occurred within 30 minutes of glutamate exposure, prior to there being a significant decrease in JIP1 protein levels, thus ruling out the possibility that the reduced binding was simply due to a reduction in JIP1 protein in the neurons (Fig. 5D). Studies in non-neuronal cells have shown that JNK phosphorylation of JIP1 controls its ability to bind to AKT (Song and Lee, 2005a; Song and Lee, 2005b), whereas in cortical neurons treated with NMDA, it is reported that inhibition of JNK activity can stabilize JIP1 (Centeno et al., 2007). This suggested that glutamate stimulation induces JNK phosphorylation of JIP1 thus causing the dissociation of the JIP1–AKT complex and subsequent destabilization of the proteins in the growth cone. If this is the case, inhibition of the JNK pathway should rescue the glutamate-induced loss of JIP1 and AKT. Indeed, when JNK activity was blocked by two different inhibitors, the glutamate-induced degradation of JIP1 and AKT was prevented (Fig. 5E–G; supplementary material Fig. S3). The major JNK phosphorylation site on JIP1 that is proposed to regulate its binding to AKT is Thr103 (Song and Lee, 2005a; Nihalani et al., 2003), so we tested whether mutation of this site to a non-phosphorylatable Ala residue prevented glutamate-induced JIP1 degradation. Glutamate stimulation did not cause a reduction in JIP1 T103A protein in the axon tips in contrast to the significant decrease in wild-type JIP1 (Fig. 5H). Furthermore, the JIP1 T103A mutant maintained AKT levels in the growth cone (Fig. 5I). Taken together, these data support a model whereby glutamate regulates the stability of JIP1 and AKT in growth cones by controlling their association through the activation of the JNK pathway.

Stabilization of JIP1 reverses the glutamate-induced block in axon growth

The knockdown of either AKT (Fig. 6A) or JIP1 (Fig. 6B) (Dajas-Bailador et al., 2008) reduces axonal growth in cultured neurons. Similarly, glutamate stimulation can slow down axon growth (Fig. 6B). However, there is no additive effect on axon growth of glutamate treatment plus JIP1 knockdown (Fig. 6B), which would be consistent with glutamate causing a slowing of axon growth by reducing JIP1 stability. The glutamate-induced decrease in axon growth could be rescued by inhibition of JNK activity (Fig. 6C), which protects JIP1 from degradation (Fig. 5E,F). Furthermore, when we expressed the stabilized JIP1 mutant (T103A) in neurons we found that, unlike wild-type JIP1, it could rescue the decrease in

Fig. 2. AKT and JIP1 protect each other from degradation by the proteasome. Cortical neurons at 4.5 d.i.v. were treated with the proteasome inhibitor MG132 (0.2 μM, 16 hours) following lentiviral shRNA mediated knockdown of AKT1/2/3 or JIP1. (A) Quantification of JIP1 fluorescence intensity in axon tips after AKT1/2/3 knockdown and MG132 treatment. (B) Quantification of AKT fluorescence intensity in axon tips after JIP1 knockdown and MG132 treatment. (C) qPCR of JIP1 and AKT mRNA in neurons expressing shRNA against AKT1/2/3. Values are means ± s.e.m. of three experiments (*P<0.05).
axon growth after glutamate stimulation (Fig. 6D). In this experiment we transfected relatively low amounts of the JIP1 expression constructs because at high levels of expression JIP1 can cause a substantial increase in axon length (Dajas-Bailador et al., 2008). These results support a model whereby glutamate-induced JNK phosphorylation of T103 in JIP1 promotes slowed axon growth. It might be expected that the reduced axon growth caused by JIP1 knockdown could be rescued by inhibition of the proteasome if JIP1 plays a role in stabilizing proteins (such as AKT) that are required for growth. Indeed, treatment of neuronal cultures with MG132 did rescue the defect in growth following JIP1 knockdown, thus supporting this hypothesis (Fig. 6E). Taken together, these results indicate that the control of JIP1 levels in the growth cone in response to neuronal activity is required for the regulation of the dynamics of axon growth.

**DISCUSSION**

The polarisation of neurons is a crucial step in the development of the nervous system and involves the integration of many signalling pathways to control cytoskeletal dynamics (Barnes and Polleux, 2009). One of the key pathways involves the lipid kinase PI3K that promotes AKT activation (Read and Gorman, 2009). The asymmetric distribution of active AKT in the developing axon tip, but not in the dendrite tips, is essential for polarisation to occur (Read and Gorman, 2009). It has previously been proposed that selective degradation of AKT by the proteasome specifically in the dendrites contributes to the asymmetric distribution of AKT (Yan et al., 2006). In this study we demonstrate that the JNK MAP kinase scaffold protein JIP1, which we have previously shown is required for polarisation and axon growth (Dajas-Bailador et al., 2008), can protect AKT from degradation in the axon tip (Figs 1, 2). Furthermore, we demonstrate a collaborative relationship between these proteins, as the stability of JIP1 requires the presence of AKT (Figs 1, 2). It is currently unclear how the asymmetric distribution of AKT and JIP1 is maintained. Our data would be consistent with a model whereby JIP1 is localised to the emerging axon tip through association with the motor protein kinesin-1 (Verhey et al., 2001; Dajas-Bailador et al., 2008; Jacobson et al., 2006), where it associates with AKT, resulting in a stabilized complex that promotes axon growth.

How JIP1 facilitates the changes in cytoskeletal architecture required to promote axon growth is also unclear. JIP1 can cooperate with fasciculation and elongation protein zeta-1 (FEZ1) to activate kinesin-1 microtubule binding and motor activity (Blasius et al., 2007), indicating that it might enhance the transport of other proteins specifically to axonal growth cones where they could maintain polarisation and promote axon growth. This is supported by studies in *Drosophila* where loss of function of the JIP1 orthologue Aplip1 leads to defects in axonal transport of vesicles and mitochondria (Horiuchi et al., 2005). Our data suggest that JIP1 is not essential for the transport of AKT to axon tips because AKT is still present in the growth cones of neurons that have been incubated with a proteasome inhibitor following knockdown of JIP1 (Fig. 2B). AKT can regulate many proteins that influence axon growth (Read and Gorman, 2009). Some of the best characterized are microtubule-associated proteins, including CRMP2 and Tau that can be targeted by AKT control of GSK3β activity. Also, AKT can directly target proteins involved in actin organization including girdin and ezrin (Read and Gorman, 2009). Therefore, regulating the stability of the JIP1–AKT complex is likely to be an important mechanism in
rapid modulation of the cytoskeleton and axon growth dynamics in response to developmental cues or neuronal activity. Furthermore, the manipulation of the JIP1–AKT axis could represent a potential route for promoting nerve regeneration. There is already evidence that JIP1 promotes regeneration of adult mouse dorsal root ganglion neurons (Barnat et al., 2010) and a number of studies have proposed a role for AKT-mediated signalling in axonal regeneration (Namikawa et al., 2000; Kim et al., 2011; Song et al., 2012).

It is becoming increasingly apparent that the regulation of protein levels constitutes an important mechanism in the modulation of neuronal function (Gallo and Letourneau, 2002; DiAntonio and Hicke, 2004). The demonstrated role of glutamate stimulation through NMDA receptors in promoting instability of AKT and JIP1 in axons may be particularly relevant since the establishment of neuronal connections in the developing brain is not only guided by specific signalling molecules and growth-promoting factors, but also by specific patterns of neuronal activity. It has been proposed that NMDA receptors can have a role in experience-dependent circuit refinement through regulating axonal structural plasticity (Gogolla et al., 2007). For example, conditional cortical knockout of the NMDA receptor component NR1 in mice leads to thalamocortical axons developing more extensive arborizations (Lee et al., 2005), and in the tadpole tectum, selective branch elimination is prevented by NMDA receptor blockade (Ruthazer et al., 2003). It is possible that the proteasomal regulation of JIP1 and AKT may contribute to the plasticity of presynaptic boutons and axon branches in the developing and adult brain.

The mechanism by which NMDA receptor signalling mediates JIP1 and AKT degradation involves the influx of calcium ions (Fig. 4H) and activation of the JNK pathway (Fig. 5E–G). It will clearly be of interest in the future to also identify the ubiquitin ligase complexes that target JIP1 and AKT for degradation. The involvement of calcium and JNK in regulating JIP1 stability is supported by other studies that demonstrate: (1) calcium added to cell lysates in vitro can promote JIP1 ubiquitylation (Allaman-Pillet et al., 2003), and (2) NMDA treatment of neurons causes a
JNK-dependent decrease in JIP1 (Centeno et al., 2007). These observations, combined with our own findings, suggest a model whereby glutamate promotes JNK-mediated phosphorylation of JIP1 leading to its dissociation from AKT and subsequent degradation of both proteins in the axon tip. This loss of JIP1 and AKT mediates glutamate-induced cessation of axon growth. Crucially, AKT protein levels in the axon tip and axon growth can both be restored by the expression of a mutant JIP1 with a key JNK phosphorylation site mutated (Fig. 5I; Fig. 6D), thus providing strong evidence that the proposed mechanism is important for regulating axonal growth. It has previously been observed that inhibition of the JNK pathway prior to polarisation can suppress axonogenesis (Oliva et al., 2006), whereas our results suggest that JNK may not be essential for axon growth following polarisation (Fig. 6C), but can play a role in modulating the response of the growth cone to glutamate stimulation.

Taken together, our study demonstrates an important collaboration between JIP1 and AKT to promote axon formation.
and provides evidence that the JIP1–AKT axis responds to neuronal activity in order to modulate axonal growth dynamics.

**MATERIALS AND METHODS**

**Neuronal cell culture**

Primary embryonic cortical neurons were obtained and cultured as described before (Dajas-Bailador et al., 2008). Briefly, cortices of E17 embryos of C57 mice were dissected in HBSS (Life Technologies Ltd, Paisley, UK) and trypsinised. Following trituration, dissociated neurons were plated in Neurobasal medium supplemented with 2% B27 and 1% GlutaMAX (all from Life Technologies Ltd), on poly-D-lysine (Sigma, Dorset, UK)-coated plates. Cell density was 5×10⁴ neurons per well in six-well plates for western blotting and immunoprecipitation experiments and 2×10⁴ neurons per well in 24-well plates, on glass coverslips, for immunocytochemistry experiments. JNK inhibitor II (SP600125) (Bennett et al., 2001) and JNK inhibitor XVI (JNK-i; 2×10⁻⁵ M, overnight) were used at a final concentration of 10⁻⁵ M, except in glutamate stimulation experiments, where it was used at 10⁻⁴ M for 30 minutes before the addition of glutamate.

**Lentiviral vector production and concentration**

HEK293T cells were co-transfected with the appropriate shuttle vector and the helper plasmids pMDLg/pRRE, pMD2.G and pRSV-Rev by calcium phosphate precipitation. Cell medium from transfected cells was collected over 2 days and centrifuged at 6000 g for 1.5 hours. The final viral pellets were re-suspended in PBS at 1:2000 of the original media volumes. Viral titres were calculated by fluorescence-activated cell sorting (detecting EGFP-expressing cells), after HEK293T cell transduction.

**Compartmentalized culture in microfluidic chambers**

Primary cortical neurons (E17) were cultured for 5 days in microfluidic devices with 150 μm long microgrooves (Xona Microfluidics LLC, Temecula, CA, USA), as described previously (Dajas-Bailador et al., 2012). The chambers allow for fluidic isolation of the axonal from the cell body (somal) compartment. Glutamate was applied only to the axonal side of the microfluidic device.

**Plasmid construction**

For shRNA vector construction, inserts were designed as complementary oligonucleotides coding for the appropriate shRNA, producing blunt ends and XhoI ends upon annealing and were ligated into HpaI–XhoI-digested pLL3.7 plasmid (Rubinson et al., 2003). Oligonucleotide sequences were: shJIP1: 5′-TGACCAACCCATCGGGCTATTCAAGGATAGCCGATGTTTCTGCTTTTTC-3′, 5′-CGAGAAAAAGAGCAAACCACATCGGGCTATCTGTTAATAGCCCGATGTTGCTGTC-3′; shAkt3: 5′-TGGTACAGTGACCACTTTCTCAAAAGAATGAGA-3′, 5′-TGCAAGAAAAGAGCACTTTCTCAAAAGAATGAGA-3′; shAkt1/2: 5′-TGCAAGAAAAGAGCACTTTCTCAAAAGAATGAGA-3′, 5′-TGCAAGAAAAGAGCACTTTCTCAAAAGAATGAGA-3′; and the helper plasmids pMDLg/pRRE, pMD2.G and pRSV-Rev by calcium phosphate precipitation. Cell medium from transfected cells was collected over 2 days and centrifuged at 6000 g for 1.5 hours. The final viral pellets were re-suspended in PBS at 1:2000 of the original media volumes. Viral titres were calculated by fluorescence-activated cell sorting (detecting EGFP-expressing cells), after HEK293T cell transduction.

**Fig. 6. Stabilized JIP1 reverses the glutamate-induced block in axon growth.** (A) Neurons at 8 d.i.v. were subjected to siRNA-mediating knockdown of AKT isoforms, and axon length measured. (B) Neurons subjected to siRNA-mediating knockdown of JIP1 were treated with glutamate (100 μM, 6 hours) or left untreated, and axon length measured. (C) Neurons incubated with or without JNK inhibitor XVI (JNK-i; 2 μM) were treated with glutamate (100 μM, 6 hours) or left untreated, and axon length measured. (D) Neurons were transfected with pEGFP-C2 and either a plasmid expressing T7-tagged JIP1 or JIP1 (T103A), and treated with glutamate or left untreated. The length of axons in GFP-positive neurons was measured. (E) Neurons were subjected to lentiviral-mediated knockdown of JIP1 (shJIP1) and were treated with MG132 (0.2 μM, 16 hours) or left untreated, and axon length was measured (*P<0.05, **P<0.01, ***P<0.001).
**Plasmid transfection and lentiviral transduction of cells**

Plasmids and JIP1 siRNA (sc-35723, Santa Cruz, Heidelberg, Germany) or AKT siRNA (sc-43610, Santa Cruz) were either electroporated into neurons using the Mouse Neuron Nucleofector Kit (Lonza Biosciences, Wokingham, UK) or transfected using Lipofectamine 2000 (Life Technologies Ltd) according to the manufacturer’s instructions. Typically, 1–1.5×10^5 neurons were electroporated with 5–10 μg of plasmid, before plating. Neurons were transduced with lentivirus on the day of plating at a multiplicity of infection (MOI) of 10 for each vector. In AKT knockdown experiments, the LV-shCTRL vector was used at MOI of 20 to control for the total viral load resulting from using both LV-shAkt1/2 and LV-shAkt3 at MOI of 10 each in the knockdown condition.

**Quantitative RT-PCR**

Total RNA was extracted using the RNasy Kit (Qiagen, Manchester, UK) according to the manufacturer’s instructions and RNA samples were treated with DNase (DNAfree Kit; Life Technologies Ltd). Quantitative PCR was performed using Multiscribe Reverse Transcriptase and SYBR-Green PCR Reaction Mix (Life Technologies Ltd) in a single-step reaction according to the manufacturer’s instructions, and run on a BioRad C1000 Thermal Cycler. The primers used were: Jip1, 5′-GCTATATTCAGGTTTGTGCCTC-3′ and 5′-GTCGCCCTCACA-TTTCGAC-3′; Akt1, 5′-GTATGAGAGAAGCTGAGGC-3′ and 5′-GATCATCTGACGTGAACCTCC-3′. Typically, 20 ng of total RNA was used per reaction.

**Antibodies**

Primary antibodies used in this study were: mouse anti-JIP1 (sc-25267; Santa Cruz), rabbit anti-AKT and anti-AKT(P-S473) (9272 and 4060; Cell Signaling) that recognize all AKT isoforms, anti-AKT1 (sc-1618, Santa Cruz), mouse anti-Tau (MAB3420; Merck Millipore), Alexa Fluor 488-conjugated anti-Tau (MAB3420A4; Merck Millipore), rabbit anti-β-tubulin and anti-β-actin (ab6046 and ab2287; Abcam, Cambridge, UK), mouse anti-t-T7 (69522; Merck Millipore), SOX1 (Abcam), mouse anti-T7 (69522; Merck Millipore), SOX1 (Abcam), mouse anti-HA, mouse FITC-conjugated anti-β-tubulin and acetylated tubulin (all from Sigma). Western blot secondary antibodies were: Odyssey goat anti-mouse IRDye 680LT and goat anti-rabbit IRDye 680LT (Li-Cor Biosciences, Cambridge, UK). Immunocytochemistry secondary antibodies were: Alexa Fluor 594 anti-mouse, Alexa Fluor 647 anti-mouse, Alexa Fluor 594 anti-rabbit, Alexa Fluor 555 anti-rabbit, and Alexa Fluor 488 anti-rabbit (all from Life Technologies Ltd).

**Immunoprecipitation**

For endogenous JIP1 and AKT pulldown experiments, 3×10^6 neurons were lysed in 300 μl 1% NP40 lysis buffer (Kim et al., 2002) containing 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanesulfonyl fluoride and 1 mM NaN3, and protein complexes were incubated overnight with 3 μg of anti-AKT1 antibody. Following antibody incubation, complexes were precipitated by incubation for 1 hour, at 4°C, with 15 μl Dynabeads Protein-G (Life Technologies Ltd).

**Immunoblotting**

Cells were lysed in TLB buffer (1% Triton X-100, 10% glycerol, 20 mM Tris-HCl pH 7.4, 137 mM NaCl, 25 mM sodium β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, plus protease inhibitors). Samples (lysatess or immunoprecipitates) were mixed with an appropriate volume of a 6x SDS loading buffer (200 mM Tris-HCl pH 6.8, 7.5% SDS, 0.5 M dithiothreitol, 30% glycerol, 0.1% Bromophenol Blue) and loaded onto a 10% SDS-polyacrylamide gel, followed by transfer onto a PVDF membrane. Membranes were blocked with 5% milk in TBS (15 mM Tris-HCl pH 7.4, 150 mM NaCl) followed by primary antibody incubation at 4°C overnight: anti-JIP1 at 1:1000, anti-AKT at 1:500, anti-β-tubulin and anti-β-actin at 1:5000. All dilutions were in 5% milk with TBS–TWEEN-20 (0.1%). Blots were then washed four times for 30 minutes with TBS–TWEEN (0.1%) and incubated for 1 hour, at room temperature with the corresponding secondary antibody at a dilution of 1:40,000 in TBS–TWEEN (0.1%) with 0.01% SDS, followed by three 10-minutes washes with TBS–TWEEN (0.1%). Proteins were detected using a Li-Cor Odyssey® System scanner according to the manufacturer’s instructions.

**Immunocytochemistry**

Neurons were fixed for 15 minutes in 4% paraformaldehyde and permeabilised in 0.2% Triton X-100 in PBS for 5 minutes, followed by blocking in 3% BSA–PBS for 1 hour. Primary antibodies were used at 1:100 (AKT) or 1:200 (JIP1) dilution (in 3% BSA–PBS), overnight at 4°C, followed by three washes with 0.1% Triton-X100 in PBS. Appropriate secondary Alexa Fluor antibodies were used at 1:250–1:500 dilution in 3% BSA–PBS for 1 hour at room temperature, followed by three washes with 0.1% Triton-X100/PBS before the cells were mounted on glass slides using Prolong Gold Antifade Reagent with DAPI (Life Technologies Ltd). In some experiments, to visualise the neurons, coverslips were incubated with FITC-conjugated anti-β-tubulin antibody (1:500 dilution) for 1 hour at room temperature, after the secondary antibody and before mounting. Images were collected on an Olympus BX51 upright microscope using a 20x/0.5 NA UPlanFLN or a 60x/0.65–1.25 NA UPlanFLN objective and captured using a CoolSnap HQ camera (Photometrics, Tucson, AZ, USA) through MetaVue Software (Molecular Devices; Sunnyvale, CA, USA). Quantification of mean fluorescence intensity of JIP1 and AKT at axon tips was performed using ImageJ (http://rsb.info.nih.gov/ij) on images obtained using the 100x objective. Images of fields of neurons were taken with the 60x objective. More than 50 axon tips were imaged per condition in each of at least three independent experiments. For the measurement of axons, an axon was defined as a neurite that was longer than 80 μm and at least three times the length of other processes. Axon length was measured using ImageJ and data expressed as the means ± s.e.m. The probability distribution of the data set was analysed before further statistical analysis (−100 axons measured for each condition from n=3 or 4). Statistical analysis of test data compared with the respective control was performed using Prism (V 5.0c) (*P<0.05, **P<0.01, ***P<0.001). Multiple group comparisons were carried out using ANOVA (post-hoc Tukey’s test) or Kruskal–Wallis one-way analysis of variance (post-hoc Dunn’s test) for non-parametric data. Pairwise comparisons were carried out using Student’s t-test or Mann–Whitney test for non-parametric analysis.

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**Competing interests**

The authors declare no competing interests.

**Author contributions**

A.J.W., F.D.B. and I.B designed research; F.D.B., I.B. and E.V.J. conducted experiments; A.J.W. and F.D.B. wrote the paper.

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**Supplementary material**

Supplementary material available online at http://jcs.biologists.orglookup/suppl/doi:10.1242/jcs.137208/-/D

**References**


**Supplementary Figure 1**

**JIP1 and AKT co-localize to the tips of axons.** Cortical neurons were fixed at 3 d.i.v and stained with anti-JIP1, anti-AKT and anti-Tau antibodies. Axon tips are indicated by white arrows. Scale-bar: 50μM.

**Supplementary Figure 2**

**Glutamate stimulation does not result in excitotoxic degeneration of neurons.** Neurons were transfected with constructs expressing either T7-tagged JIP1 or JIP1(T103A) and stimulated with or without glutamate (Glut) (100μM, 3hrs). Nuclear integrity was assessed by DAPI staining. Approximately 200 neurons per condition from 4 independent experiments were analysed.

**Supplementary Figure 3**

**Glutamate regulates JIP1 and AKT stability via JNK.** Neurons were stimulated with glutamate (Glut) (100μM, 3hrs) after pre-treatment with JNK inhibitor II (10μM) for 30 mins and the fluorescence intensities of JIP1 (A) and AKT (B) immunostaining in the axon tips was quantified. (*p < 0.05).