The effects of short-term JNK inhibition on the survival and growth of aged sympathetic neurons.

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Abstract

During the course of normal aging, certain populations of nerve growth factor (NGF)-responsive neurons become selectively vulnerable to cell death. Studies using dissociated neurons isolated from neonates have shown that c-Jun N-terminal kinases (JNKs) are important in regulating the survival and neurite outgrowth of NGF-responsive sympathetic neurons. Unlike neonatal neurons, adult sympathetic neurons are not dependent on NGF for their survival. Moreover, the NGF precursor, proNGF, is neurotoxic for aging but not young adult NGF-responsive neurons. Because of these age-related differences, the effects of JNK inhibition on the survival and growth of sympathetic neurons isolated from aged mice was studied. Aged neurons, as well as glia, were found to be dependent on JNK for their growth but not their survival. Conversely, proNGF neurotoxicity was JNK-dependent and mediated by the p75-interacting protein NRAGE, while neurite outgrowth was independent of NRAGE. These results have implications for the potential use of JNK inhibitors as therapies for ameliorating age-related neurodegenerative disease.

Keywords

Aging, JNK, neurons, NGF, ProNGF, NRAGE

1. Introduction

Normal aging is often accompanied by deficits in the autonomic nervous system, impacting on, for example, gastrointestinal function (Camilleri et al., 2008), control of temperature (Greaney et al., 2016), cardiac activity (De Meersman and Stein, 2007) and blood pressure (Monahan, 2007). Such deficits, all of which impair quality of life, can result from neuron loss, e.g. of enteric neurons (Camilleri et al., 2008). Understanding the mechanisms of age-related neurodegeneration may permit interventions to prevent such effects.

During normal aging, sub-populations of NGF-responsive neurons become susceptible to cell death in both the central nervous system (CNS) (Geula et al., 2003; Mufson et al., 1989; Mufson et al., 2000; Smith et al., 2004) and in the peripheral nervous system (PNS) (Andrews et al., 1996; Gatzinsky et al., 2004). NGF-responsive PC12 cells (Greene and Tischler, 1976) and NGF-dependent neonatal neurons (Chun and Patterson, 1977) have been used extensively as models to study the molecular pathways that affect neuronal survival and neurite outgrowth in neurodegenerative disease. Although important insights can be gained from such studies, there are important differences in the survival characteristics between neurons from developing animals and those from aged adults. Embryonic and early post-natal sympathetic neurons derived from the superior cervical ganglion (SCG) differ from adult neurons in that they are NGF-dependent for their survival (Chun and Patterson, 1977), and withdrawal of NGF leads to apoptosis (Martin et al., 1992). Adult SCG neurons, on the other hand, are able to survive without NGF but, like developing neurons, are NGF-dependent for neurite growth (Orike et al., 2001a; Orike et al., 2001b). Moreover, there are also important differences in the response of neurons from young adult and aged animals to the NGF precursor proNGF, the main form of the protein in the adult nervous system (Bierl and Isaacson, 2007; Fahnestock et al., 2001). ProNGF promotes neuritogenesis of young adult neurons (Al-Shawi et al., 2008), but can be neurotoxic to NGF-responsive neurons of the aged PNS and CNS (Al-Shawi et al., 2008; Jansen et al., 2007).

NGF promotes survival and neurite outgrowth through its receptor TrkA (Chao and Hempstead, 1995); this activity is enhanced when TrkA binds p75 to form a high affinity complex for NGF (Esposito et al., 2001). In contrast, proNGF binds to the receptors sortilin and p75 to form a pro-apoptotic complex

(Nykjaer et al., 2004). Aged neurons are more susceptible to proNGF/p75/sortilin-mediated cell death and this correlates with increased levels of some forms of proNGF as well as the receptor sortilin (Al-Shawi et al., 2008; Jansen et al., 2007).

The low affinity NGF receptor p75 elicits different cellular responses, depending on its interaction with co-receptors and other proteins which bind to its intracellular domain (Gentry et al., 2004). NRAGE (neurotrophin receptor-interacting MAGE homologue) is a cytoplasmic protein that has been implicated in p75-promoted apoptosis of NGF-dependent neurons (Salehi et al., 2000; Salehi et al., 2002). Consistent with a role of NRAGE in p75-mediated apoptotic signalling, sympathetic neurons from neonatal NRAGE knockout mice are resistant to BDNF/p75-mediated cell death (Bertrand et al., 2008). NRAGE-mediated apoptosis of NGF-responsive cells is dependent on c-Jun N-terminal kinase (JNK) activity, and JNK inhibition rescues neurons from NRAGE-mediated cell death (Salehi et al., 2006; Salehi et al., 2002). However, it is not known if proNGF/p75/sortilin-mediated cell death of aged NGF-responsive neurons is also mediated by NRAGE and JNK.

JNKs are important in regulating growth and apoptosis in the CNS and PNS and have important roles in the formation and differentiation of the developing nervous system and in the responses of the adult nervous system to injury or biochemical insult. They are activated in response to many stimuli, including cytokines and growth factors. JNK1 and JNK2 are present in most tissues and are important for regulating apoptosis in the developing nervous system (Kuan et al., 1999), while JNK3 is mainly restricted to the nervous system (Martin et al., 1996) and has been found to be an important mediator of cell death following brain injury in both adults and neonates (Pirianov et al., 2007).

Although JNKs have a clearly established role as mediators of pro-apoptotic signalling, it is clear that they are also important in the regulation of neurite outgrowth during normal development and in regeneration of the damaged adult nervous system (Barnat et al., 2010; Kenney and Kocsis, 1998; Lindwall et al., 2004; Ruff et al., 2012). The survival and differentiation of glia is also regulated by JNKs in both the CNS (Casaccia-Bonnefil et al., 1996; Jurewicz et al., 2003; Pirianov et al., 2006; Yoon et al., 1998) and PNS (Parkinson et al., 2004).

JNK activation has been implicated in age-related neurodegenerative diseases such as Alzheimer's disease (Killick et al., 2014; Morishima et al., 2001; Savage et al., 2002; Vogel et al., 2009; Zhu et al., 2001), Parkinson's disease (Brecht et al., 2005), in degenerating motoneurons (Newbern et al., 2007) and neurodegeneration in the PNS (Gomez-Sanchez et al., 2015). Such considerations have led to the investigation of JNKs as potential therapeutic targets for neuroprotection (Antoniou et al., 2011; Bogoyevitch et al., 2010; Manning and Davis, 2003).

In order to take account of changes that occur during aging, in this study we investigated the effects of JNK inhibition on the survival and growth of NGF-responsive sympathetic neurons isolated from aged animals.

2. **Materials and Methods**

2.1 *Animals*

For the majority of studies, young adult (3-6 months old) and old (24-36 months old) C57BL/6 x CBA/Ca male mice or Sprague-Dawley rats were used and maintained as previously described (Al-Shawi et al., 2008). For some experiments, C57BL/6 young (3-6 months old) and old (24-36 months old) male mice were compared. All animal studies were ethically reviewed and approved by the UCL Royal Free Campus Ethics and Welfare Committee and the UK Home Office, and were carried out in accordance with European Directive 86/609/EEC.

2.2 *Dissociated cell cultures of SCG neurons*

Dissociated cultures of adult and aged mouse neurons were prepared and counted essentially as previously described (Al-Shawi et al., 2008; Orike et al., 2001b). Dissociated cells were cultured in neurobasal-A medium with B27 supplement (Gibco, UK), in the presence of 3μM cytosine arabinofuranoside (AraC; Sigma, UK) for 24 hours unless otherwise indicated. Neurons were plated on collagen IV (Sigma, UK) coated coverslips. Neonatal neurons were isolated and cultured in the presence of 1.9nM NGF (Nykjaer et al., 2004) for 24h prior to starting experiments. They were then washed twice in NGF–free medium, before replacing the medium with that for experimental and control conditions. Neurons were counted as previously (Al-Shawi et al., 2008; Orike et al., 2001a) using phase contrast

optics at 100 X magnification. Neurons that were alive were identified by their large phase-bright, intact cell bodies and neurites (Orike et al., 2001a), and were very clearly distinguishable from dead neurons which were dark with fragmented soma and neurites and which were less firmly attached to the substrate. To avoid trophic effects from other neurons or glia, cultures were seeded at low density, and neurons whose cell bodies were in contact with other neurons or glia were excluded. Neurons were scored as growing only if extended neurites were longer than twice the diameter of the cell soma (Orike et al., 2001b). Because of the inherent variability in the survival of un-treated control neurons isolated from aged mice, results from representative single experiments are shown as these are internally consistent. Experiments were repeated in triplicate and results were only deemed to be significant if there was concordance between repeats. Typically, after 24h in culture under control conditions, surviving neurons comprised 50-80% of those plated, 30-40% of which grew when NGF- or proNGF-stimulated.

To allow cultures to be followed for more than 24 hours, the nucleoside analogue, cytosine arabinofuranoside (AraC) was included in the culture medium at 3µM, to inhibit glial proliferation, which would otherwise compromise counting. Although lower than previously used for SCG cultures (10µM (Orike et al., 2001a)) this concentration was equally effective in inhibiting glial proliferation, without adversely affecting neuronal survival and growth in control cultures (supplementary fig. 1). AraC was omitted in studies of glial survival and growth. Glia were identified by their characteristically small and spindle-shaped phase-grey cell bodies. Cell-type identity was confirmed by immunohistochemical analysis: glia were GFAP positive and Tuj-1 negative (supplementary fig. 2). To assess viability, Molecular Probes Live/Dead Viability/Cytotoxicity assay kit was used to determine the ratio of live/dead cells following the manufacturer's instructions.

Nerve growth factor (NGF, murine 2.5S) was purchased from Promega, UK. ProNGF was purchased from Axxora, Germany (human recombinant proNGF) and Alamone labs, Israel (furin cleavage-resistant proNGF (mutated-mouse) N-255). In adult neuron cultures, NGF and proNGF were used at 0.4nM, similar to the physiological concentrations measured in adult rat SCG and iris (Cowen et al., 1996), and previously found to support robust growth of rat SCG neurons (Al-Shawi et al., 2008; Orike et al., 2001a).

Experiments investigating proNGF neurotoxicity were carried out in insulin free medium (neurobasal-A medium supplemented with insulin-free B27), in order to eliminate effects of insulin on survival and growth, as previously (Al-Shawi et al., 2008).

JNK inhibitor II (anthra[1,9-cd]pyrazol-6(2H)-one), also known as SP600125, JNK inhibitor III (Ac-YGRKKRRQRRR-gaba-ILKQSMTLNLADPVGSLKPHLRAKN-NH_2), JNK inhibitor VIII (N-(4-Amino-5-cyano-6-ethoxypyridin-2-yl)-2-(2,5-dimethoxyphenyl)acetamide), JNK inhibitor IX (N-(3-Cyano-4,5,6,7-tetrahydro-1-benzothien-2-yl)-1-naphthamide) and AEG3482 (6-Phenylimidazo[2,1-b]-1,3,4-thiadiazole-2-sulfonamide) were all purchased from Calbiochem (UK). All were used at a final concentration of 10µM, and were included in the culture medium at the time of plating. The choice of concentration was informed by published information about their potency in cell-based assays, as well as precedent (Angell et al., 2007; Bennett et al., 2001; Gao et al., 2013; Holzberg et al., 2003; Salehi et al., 2006; Szczepankiewicz et al., 2006; Vivanco et al., 2007). With competitive inhibitors (e.g. JNK inhibitors II, VIII and IX), significantly higher concentrations of inhibitor are required for cell-based experiments than for biochemical assays on purified proteins because of the high ATP concentration within cells and/or active efflux of these compounds from cells. JNK inhibitor III, a pan-JNK inhibitor which disrupts the JNK-c-Jun interaction was used at a 10-fold lower concentration than originally reported (Holzberg et al., 2003) to avoid non-specific effects. Neuronal survival and growth were followed for up to 72 hours. Similar survival and growth rates relative to controls were obtained for consecutive days, and all results presented are for 24 hours of culture, unless otherwise stated.

For small interfering RNA (siRNA) knock-down, siRNAs (Dharmacon, UK) were linked to the vector peptide Penetratin-1 (Davidson et al., 2004) (MP Biomedicals, UK) as directed by the supplier. The sequences for NRAGE siRNAs were as described and validated by (Matluk et al., 2010). siRNAs were administered at 80nM.

Because of the low levels of NRAGE expression and the impracticality of obtaining sufficient material from isolated neuronal cultures for Western blot analysis of NRAGE protein expression, the efficacy of NRAGE knock-down after coupling the siRNAs to Penetratin-1 was confirmed by (1) its ability to rescue neonatal SCG neurons from NRAGE-mediated cell death after NGF withdrawal (Salehi et al., 2006) and

(2) immunostaining of adult aged SCG neurons after treatment for 24 hours with coupled control and NRAGE siRNAs (supplementary Fig. 3).

2.3 *Western blot analysis*

Western blot analysis was carried out essentially as previously described (Al-Shawi et al., 2008). Brain samples were homogenised in cold HB buffer. SCG samples were sonicated in HB on ice, by six one second bursts at an amplitude of 10 microns. HB buffer consisted of 200mM Tris pH7.4, 2mM EDTA pH7.4, 2mM EGTA pH 7.4, 1mM PMSF, 0.5% triton X-100 and 1 complete mini protease inhibitor cocktail tablet (Roche Diagnostics; Mannheim, Germany) per 10 ml. Anti-NRAGE primary antibodies were used at 1:500 (rabbit polyclonal, Upstate, UK) and 1:2000 (rabbit polyclonal, Santa-Cruz, UK). Primary antibody incubations were overnight at 4°C. To control for variation in protein loading, Western blots were stripped prior to re-probing with anti-actin (clone 4 MAB1501, Millipore, UK) at a dilution of 1:2000. Secondary antibodies were used at 1:1000 (goat anti-rabbit Ig-HRP, Dako, UK) or 1:2000 (rabbit anti-mouse Ig-HRP, Dako, UK) for 1 hour at room temperature. Signal was detected using ECL substrate and Hyperfilm ECL (GE healthcare, UK), taking care to avoid overexposure.

2.4 *In situ immunostaining*

For immunofluorescence analysis, cells were washed in PBS, and fixed for 10 minutes with 4% paraformaldehyde before proceeding with the protocol previously described (Al-Shawi et al 2008). Anti-NRAGE rabbit polyclonal (Upstate, UK) was used at 1:200, anti-GFAP rabbit polyclonal (Dako, UK) at 1:1000, and Tuj-1 anti-neuron-specific Class III β-tubulin mouse monoclonal (a kind gift of Tim Cowen) at 1:1000. Hoechst 33342 was used as a nuclear counterstain, at 1µg/ml.

Immunoperoxidase staining was carried out on 2µm wax sections of mouse SCG. Endogenous peroxidases were blocked with 0.6% H_2O_2 for 15 minutes. After blocking with 2.5% normal horse serum (Vector Laboratories, UK) for 30 minutes, anti-NRAGE antibody (1:100; Upstate, UK) was applied overnight at 4°C. Detection was with horse anti-rabbit ImmPRESS HRP polymer (Vector Labs, UK; undiluted, 30 mins.) and DAB/metal concentrate 1:10 in stable peroxide substrate buffer (Thermo Scientific, UK). Counterstaining was with Mayer's hematoxylin (Pioneer Research Chemicals, UK).

2.4 *Statistical analysis*

For analysis of survival, neurons were classified as alive or dead; for analysis of growth, dead neurons were excluded, and living neurons were classified as growing or non-growing. Statistical analysis was performed using the χ^2 test using Graphpad Prism 5.0, which evaluates the statistical significance of differences in frequencies of classes (e.g. live and dead) between samples. All comparisons were within experiment, i.e. limited to treatments and untreated control populations of cells derived from a single preparation of neurons. Threshold P-values adjusted for multiple comparisons are given in the figure legends. Experiments were repeated in triplicate and differences were not deemed to be significant unless there was concordance between the different experiments.

Quantitative analysis of western blot data was performed by densitometry of entire bands, with subtraction of background immediately adjacent to the band, and avoiding obvious artefacts where present. The ratios of background-subtracted NRAGE and actin signals from samples analysed on the same blot were analysed by the Mann-Whitney test using Graphpad Prism 5.0 to determine any significant difference in NRAGE protein, with the threshold value p=0.05.

Quantitative immunofluorescence was performed exactly as previously detailed (Al-Shawi et al., 2008); statistical analysis was by the Mann-Whitney test using Graphpad Prism 5.0, with the threshold value p=0.05.

3. Results

3.1 *JNK is required for neurite outgrowth of aged SCG neurons.*

The effect of a well-characterised pan-JNK inhibitor, JNK Inhibitor II (SP600125), on the growth of neurons isolated from the SCGs of young adult and aged adult mice was examined. This inhibitor did not significantly affect survival either in the presence or absence of NGF, (Fig. 1A,B). However, JNK Inhibitor II greatly reduced neurite outgrowth of neurons isolated from both young and old adult SCGs (Fig. 1B). These results are consistent with the observation that, unlike neonatal neurons which undergo JNK-dependent apoptosis on NGF withdrawal, adult neurons are not dependent on NGF for their survival but are dependent on NGF for their growth (Orike et al., 2001b).

Next, the effect of other JNK inhibitors on adult neurons was examined. Because adult SCG neurons are not dependent on NGF for their survival and because the effects of JNK inhibition on survival were similar in the presence or absence of NGF (Fig 1A,B and data not shown) the data shown below are confined to neurons cultured in the presence of NGF. JNK inhibitor III is a pan-JNK inhibitor which acts by disrupting c-Jun/JNK complex formation, in contrast with the JNK inhibitor II, which is a competitive inhibitor of the kinase activity. Like JNK inhibitor II, JNK inhibitor III restricted neuritogenesis of adult SCG neurons (Fig. 1C), without changing their survival, supporting a role of c-Jun in neurite outgrowth (Dragunow et al., 2000; Lindwall and Kanje, 2005).

AEG3482 is a cell-permeable imidazothiadiazole, and was identified for its ability to inhibit p75/NRAGE/JNK-mediated apoptosis in NGF-responsive neurons (Salehi et al., 2006). It was therefore of interest to examine the effect of this compound on neurite outgrowth of adult dissociated neurons. In line with the results presented above, AEG3482 showed a marked effect on neurite outgrowth of neurons of both young and aged mice, but not survival (Fig. 1D).

To explore which specific JNKs were responsible for compromising neurite outgrowth, we used more selective JNK inhibitors. JNK inhibitor VIII inhibits JNK1 and JNK2 more effectively than JNK3, while JNK inhibitor IX inhibits JNK2 and JNK3, but not JNK1. Both inhibitors gave similar levels of survival when compared with control untreated cultures (Fig. 2A). Neurite growth was restrained by inhibitor VIII but completely repressed by inhibitor IX (Fig. 2B & Fig. 3) suggesting that JNK3 is especially important for neurite outgrowth of adult and aged NGF-responsive neurons.

3.2 *JNK is required for glial outgrowth.*

In the experiments described above, we noted that JNK inhibitors appeared to affect the growth of glial extensions as well as their proliferation. Unlike neurons, glia are not post-mitotic cells, and they proliferate when explanted in culture. Because the cultures contained AraC to suppress the proliferation of glia, it was possible that the phenotypes of the glia were altered by this treatment. To clarify the roles of JNKs in glia without this complication, we prepared cultures in which AraC was omitted. In control cultures, glia proliferated profusely, and grew extensive processes. In contrast, AEG3482 and pan-JNK

inhibitor II each arrested glial proliferation and repressed the growth of glial extensions, whereas the effects of JNK inhibitor III were much less pronounced (Fig. 4). Also, whereas JNK inhibitor VIII caused some restriction of growth, in cultures treated with JNK inhibitor IX, glia appeared shrunken, with growth of processes being completely abolished. In order to determine whether this appearance reflected cell death or a profound effect on growth without cell death, the Live/Dead assay was used. The results confirmed that despite the drastic change in morphology, the cells remained metabolically active, showing that JNK inhibitor IX did not affect glial survival. Taken together, these observations suggest that, as with neurons, JNK3 is likely to be particularly important for the growth of glial cells.

3.3 *JNK and NRAGE are required for proNGF-mediated neurotoxicity*

ProNGF is neurotoxic under certain conditions, and unlike young adult neurons, some NGF-responsive aged neurons are vulnerable to proNGF-mediated cell death (Al-Shawi et al., 2008; Jansen et al., 2007). Because AEG3482 rescues NGF-responsive cells from NRAGE-mediated cell death through JNK (Salehi et al., 2006), we investigated whether the deleterious effects of proNGF on aged neurons were also mediated by NRAGE and JNK. Addition of AEG3482 to the medium rescued aged SCG neurons from proNGF-mediated cell death suggesting that JNK was required for proNGF neurotoxicity (Fig. 5A). This was confirmed when neurons were cultured with JNK inhibitors VIII or IX in the presence of proNGF although JNK inhibitor IX was found to be a more potent inhibitor than JNK inhibitor VIII. Notably, as well as protecting the neurons from proNGF-mediated cell death, JNK inhibitors VIII and IX abolished the neurite outgrowth-promoting activity of proNGF (Fig. 5B, C and data not shown).

Next, the effect of NRAGE knockdown on the survival of aged neurons treated with proNGF was studied. ProNGF-mediated neurotoxicity of aged neurons was abolished by NRAGE siRNA treatment (Fig. 5B), while neuritogenesis was not affected by NRAGE siRNA (Fig. 5C). The effects of addition of JNK inhibitor IX together with NRAGE siRNA were not significantly different from those of treatment with JNK inhibitor IX alone. These data suggest that proNGF-mediated neurotoxicity of vulnerable aged SCG neurons is both JNK–dependent and mediated through NRAGE.

3.4 *Expression of NRAGE in the aged nervous system*

Following from the observation that NRAGE is required for proNGF neurotoxicity of vulnerable aging neurons, and because NRAGE is a potential drug target (Rochira et al., 2011), we wished to determine if neuronal expression of NRAGE changed in areas of the nervous system that are vulnerable in aging. Western blot analysis was used as NRAGE expression is confined to neurons (Barrett et al., 2005) (supplementary figs. 3 & 4). NRAGE expression was found to be low in the adult nervous system, as previously observed by others (Kendall et al., 2002; Salehi et al., 2000). Because of the low levels of NRAGE expression and the limited amounts of material, we pooled SCGs from groups of 3 mice. Because this approach can obscure inter-individual variability, we also analysed material from individual rats. Pooled mouse SCG lysates and lysates from individual rats, showed considerable variation, and no consistent difference was found between NRAGE expression in young and aged animals when normalised against actin expression (Fig. 6A & 6B).

In the CNS, NGF-responsive neurons of the Nucleus basalis of Meynart (NBM) are highly vulnerable in Alzheimer's disease, and we have previously shown that mouse neurons from this region of the brain are susceptible to age-related loss, and are sensitive to proNGF-mediated cell death (Al-Shawi et al., 2008). Mouse NBM is not a morphologically discrete entity and due to its small size, it is not amenable to Western blot analysis. Quantitative image analysis was therefore carried out following immunofluorescent staining of NRAGE in NBM sections. This showed significantly higher immunoreactivity in NBM neurons of aged mice (Fig. 6E).

Western blot analysis was also used to study changes in NRAGE expression in the cortex and hippocampus, areas to which NGF-responsive neurons from the basal forebrain project, and which are vulnerable to p75-mediated cell death after seizure (Choi and Friedman, 2014; Troy et al., 2002) or ischemia (Park et al., 2000). Two different strains were examined, outbred CD1 mice, and to minimise variation between individuals, F1 offspring produced from a cross between two inbred strains of mice (C57BL/6 x CBA/Ca). In both strains, expression in the cortex was found to be moderately but significantly elevated during aging, being two and three times higher in aged F1 ($p= 0.027$; $n=5$) and CD1 ($p=0.02$; $n=4$) mice, respectively (Fig. 6. C). In mouse hippocampus, expression levels were not

found to be significantly different between young and old mice, in either strain (Fig. 6. D). These results indicate that NRAGE expression is highly variable in the adult nervous system, but also that there is higher NRAGE expression in some vulnerable parts of the aging mouse nervous system.

4. Discussion

The functional activities of JNKs in the nervous system are complex and context dependent, and neurons and glial cells of the same developmental stage can exhibit regional variability in the functional activities of different JNKs. For example, while survival of neonatal sensory neurons is reduced by JNK inhibition in the presence of NGF, the survival of similarly cultured sympathetic neurons from the same developmental stage is unaffected (Lindwall and Kanje, 2005). JNKs mediate neuritogenesis in both the PNS and CNS (Eminel et al., 2008; Lindwall et al., 2004) during normal development and in injury models (Brecht et al., 2005; Waetzig and Herdegen, 2003), but not all neurons respond equivalently, as illustrated by the observation that neurite outgrowth is JNK–dependent for early postnatal hippocampal neurons but not for cortical neurons (Eminel et al., 2008).

JNKs also have important functions in glia; they control cell proliferation and survival of Schwann cells (Parkinson et al., 2004; Woodhoo et al., 2009), and JNK activation has been found to favour Schwann cell dedifferentiation during nerve regeneration (Monje et al., 2010). In the CNS, JNKs are implicated in the regulation of proliferative and terminally differentiated oligodendrocytes (Casaccia-Bonnefil et al., 1996; Jurewicz et al., 2003; Pirianov et al., 2006; Yoon et al., 1998).

Short-term JNK inhibition does not adversely affect the survival of aged adult neurons, but does inhibit their growth. Furthermore, JNK inhibition had a profound effect on glia isolated from the SCGs of aged adult mice, halting their proliferation and markedly reducing the growth of glial extensions. The use of JNK inhibitors with different specificities, suggests that in the aged sympathetic nervous system, JNK3 is the predominant regulator for the outgrowth of both neurons and glia with JNK1 and and/or 2 having lesser effects. Similarly, the responses of neurons treated with proNGF to the different JNK inhibitors are most simply explained by JNK3 being key for both survival and neurite outgrowth. Of note, previous

studies have shown that JNK3 is implicated in the p75 mediated apoptosis pathway of NGF-withdrawn neonatal neurons (Bruckner et al., 2001; Kenchappa et al., 2010).

ProNGF-mediated apoptosis through p75/sortilin is also context dependent and is likely to be determined by availability of both neurotrophins and cell surface receptor expression (Al-Shawi et al., 2008; Masoudi et al., 2009). For example when PC12 cells are cultured under conditions in which the p75:TrkA ratio is increased, they become vulnerable to proNGF-mediated cell death (Masoudi et al., 2009). Aged NGF-responsive neurons are also vulnerable to proNGF-mediated cell death (Al-Shawi et al., 2008; Jansen et al., 2007) and as well as being exposed to increased levels of proNGF in vivo (Bierl and Isaacson, 2007), they express higher levels of sortilin (Al-Shawi et al., 2008), which acts with p75 as a co-receptor for proNGF (Nykjaer et al., 2004); SCG sympathetic neurons of sortilin knockout mice are protected from age-related cell death (Jansen et al., 2007).

A striking finding reported here is that JNK inhibition rescued aged neurons from proNGF-mediated cell death, while also attenuating or halting the growth of surviving neurons. To understand the reasons underlying the seemingly antagonistic effects on survival and growth, it may be important to note that the SCG contains a number of different population of neurons, which project to different targets. These each have distinctive morphologies, neurochemical properties and vulnerabilities (Andrews et al., 1996). During normal aging in the rat, cerebral blood vessel-projecting neurons are preferentially lost, while iris-projecting neurons are relatively spared (Andrews et al., 1996; Gatzinsky et al., 2004). In cultures of SCG neurons derived from aged rodents, only a proportion of neurons are killed by proNGF. It is likely that some neurons, including cerebral blood vessel projecting neurons, have a “less favourable” complement of cell surface receptors, making them more vulnerable to proNGF-mediated cell death. In this case, proNGF binding to p75/sortilin leads to the activation of an NRAGE-dependent pro-death pathway. In neurons with a “more favourable” receptor complement, proNGF would signal through p75/TrkA, promoting survival pathways (via mitogen-activated protein kinases, phosphoinositide 3-kinase and phospholipase-C), facilitating neurite regeneration by c-Jun-mediated transcriptional changes (Dragunow et al., 2000; Lindwall and Kanje, 2005), and allowing the cytosolic pool of activated JNK to promote neurite outgrowth, through modulation of cytoskeletal proteins (Chang et al., 2003;

Coffey et al., 2000; Gdalyahu et al., 2004; Tararuk et al., 2006). With pharmacological inhibition of JNK activity, more vulnerable neurons that have a “less favourable” receptor complement would be protected, while neurite outgrowth of all surviving neurons would be inhibited.

The p75 interacting protein NRAGE, a potential drug target (Rochira et al., 2011), has been implicated in neurotrophin/p75/JNK-mediated cell death (Salehi et al., 2006; Salehi et al., 2002), including SCG neurons (Bertrand et al., 2008), and NRAGE expression is observed in the period of naturally occurring cell death (Kendall et al., 2002). The role of NRAGE in neuronal growth is less clear; in PC12 cells, both growth-suppressing (Feng et al., 2010) and growth-promoting effects have been reported (Reddy et al., 2010), while in NRAGE knockout mice neuronal growth was found to be normal (Bertrand et al., 2008). The results presented here show that NRAGE protein expression is not changed in aged SCG, but is elevated in regions of the aged CNS that are vulnerable to age-related neuronal loss. The regional variability, along with the variability between individuals suggests that NRAGE expression is highly plastic. In this study, aged neurons were protected from proNGF-mediated cell death by NRAGE knockdown, but the same treatment had no effect on neuritogenesis. The compound AEG3248 inhibits NRAGE/JNK mediated cell death by binding to HSP90 and inducing the heat shock protein transcription factor HSF-1, and it is known that some heat shock proteins are able to block JNK activation (Salehi et al., 2006). It is therefore likely that AEG3248 is able to protect aged neurons from proNGF-mediated cell death through the upregulation of heat shock proteins.

In conclusion, there appears to be a balance between survival and growth. Of interest here, is the observation that often in neurodegenerative disease or following neurological insult, many neurons undergo atrophy rather than cell death e.g.(Kordower et al., 1989), an interesting parallel with the consequences of JNK inhibition. A therapeutic ambition is to restore function by promoting re-growth of atrophied neurons; the major challenge will be to achieve this while preserving their viability. The results presented here have implications for the potential use of JNK inhibitors to treat neuronal injuries and disease in the elderly and suggest that JNK inhibition may be useful for acute treatments to inhibit neuronal cell death and, perhaps, gliosis, but that longer periods of treatment might ultimately prevent regeneration, outgrowth and function.

Figure Legends

Fig. 1. Effects of pan-JNK inhibition on adult and old-aged sympathetic neurons.

Young adult and old-aged SCG neurons respond in a similar manner to JNK inhibition. Relative survival (filled bars) and growth (open bars) of neurons from young (A, C & D) or old-aged (B, C & D) mice is shown after culture under the conditions indicated. For clarity, survival was normalized to the control cultures; growth was normalized to NGF-containing control cultures. Young adult (A) and old-aged (B) SCG neurons are NGF-independent for their survival, but NGF-dependent for growth. Inhibitor II (A, B), Inhibitor III (C) and AEG3248 (D) all inhibit growth, but not survival on short-term culture. All cultures in (C) & (D) were supplemented with NGF. N=300. Threshold P-value = 0.0083.

Fig. 2. Effects of selective JNK inhibition on old-aged sympathetic neurons.

The growth of both young (A) and aged (B) SCG neurons in the presence of NGF is compromised by JNK inhibitor VIII and completely blocked by JNK inhibitor IX. Relative survival (filled bars) and growth (open bars) of neurons from young (A) or old-aged (B) mice is shown after culture under the conditions indicated. NGF was included in the medium for all cultures. (A) n=300, (B) n=900. Threshold P-value = 0.0167

Fig. 3. Phase contrast images of aged neurons treated with JNK inhibitors.

(A) A typical growing neuron cultured under control conditions, with NGF. (B) Neurons cultured in the presence of NGF and JNK inhibitor II, showing two living neurons and one fragmented/dead neuron (black arrow). (C) A neuron growing in the presence of NGF and JNK inhibitor III; the white arrow indicates a glial cell. (D) Neurons cultured in the presence of NGF and AEG3248, two of which show no growth and one (arrowed) is fragmented/dead. (E) Neurons cultured in the presence of NGF and JNK inhibitor VIII, one of which is dead (black arrow); the white arrow indicates a glial cell. (F) Neurons cultured in the presence of NGF and JNK inhibitor IX, showing the typical complete absence of any significant growth.

Fig. 4. Effects of JNK inhibition on growth of glia.

Representative phase contrast images of glia from old-aged mice after culture with JNK inhibitors for 72h. (A) NGF control, (B) NGF plus inhibitor III, (C) NGF plus AEG3482, (D) NGF plus inhibitor II, (E) NGF plus inhibitor VIII and (F) NGF plus inhibitor IX. Live-dead assay (G) demonstrated that the survival of inhibitor IX-treated glia is no different from that of glia cultured in the presence of NGF alone, despite the dramatic effect on growth and the unhealthy appearance of the cells.

Fig. 5. Differential effects of JNK inhibition and NRAGE knockdown on proNGF-mediated survival and growth of old-aged sympathetic neurons.

The effects JNK inhibitors and NRAGE siRNA on the survival of proNGF treated old-aged neurons. Relative survival (filled bars) and growth (open bars) of neurons from old-aged mice is shown after culture under the conditions indicated. For clarity, survival was normalized to the control cultures (A & B); growth was normalized to proNGF-treated cultures (A & C). Treatment with proNGF alone reduces the survival of old-aged SCG neurons (A & B), an effect which is blocked by treatment with AEG3248 (A), JNK inhibitor IX, or NRAGE siRNA (B). ProNGF treatment promotes growth of surviving neurons (A & C); while this is counteracted by AEG3248 (A) and JNK inhibitor IX (C), NRAGE siRNA does not inhibit proNGF-stimulated growth when compared with control siRNA. N=900. Threshold P-value for data shown in (A) = 0.0083; threshold P-value for data shown in (B) and (C) = 0.005.

Fig. 6. Age profile of NRAGE expression.

Quantitative western blot (A-D) and immunofluorescence (E) analysis of NRAGE expression in peripheral and central neuronal populations. In (A-D) the top panels show NRAGE signal, and the bottom panels show actin, as loading controls. (A) Mouse SCG. Each lane represents pooled SCGs from three individual mice. (B) Rat SCG. Each lane contains protein extracted from the SCGs of an individual rat. (C) Mouse cortex. Each lane contains protein extracted from the cortex of an individual mouse. (D) Mouse hippocampus. Each lane contains protein extracted from the hippocampus of an individual mouse. (E) Immunohistochemical analysis of NRAGE expression in mouse nucleus basalis

of Meynert and its quantitation (n=5 mice per group). GV, intensity, grey value/μm^2 derived from 99-135 optical section profiles of individual neurons per mouse; Y, young; O, old-aged, C, standard sample of control whole mouse brain lysate. Threshold P-value = 0.05.

Disclosure statement

Conflicts of interests: none

Acknowledgements

We are grateful to Tim Cowen for advice, encouragement and discussions. This work was supported by grants from the NC3Rs to JPS, the Wellcome Trust (grant no. 047310 to R.A. and 067727 to J.P.S.), the Royal Free London NHS Foundation Trust (RA, JPS) and the Biogerontology Research Foundation (JPS, RA). Core support for the Wolfson Drug Discovery Unit is provided by the UK National Institute for Health Research Biomedical Research Centre and Unit Funding Scheme.

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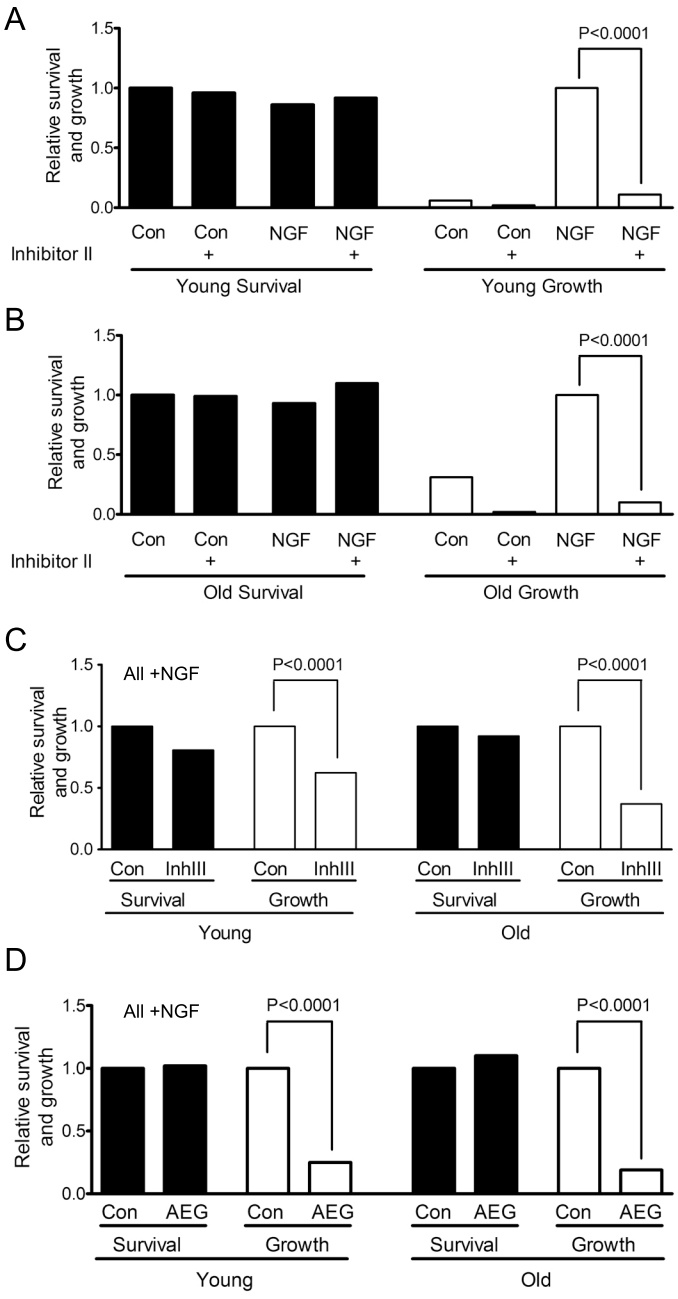
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B
Relative survival and growth
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Growth
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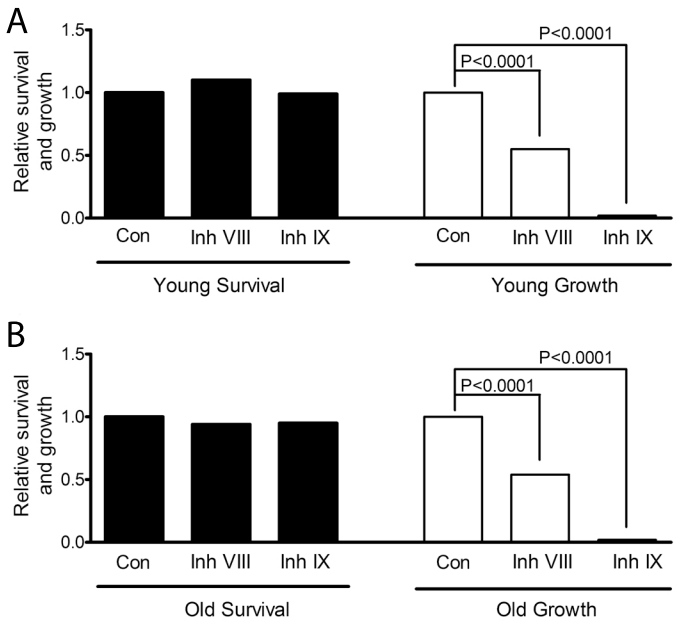
Growth
Young
Old

Figure 1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Young Survival
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B
Relative survival and growth
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Old Growth

Figure 2

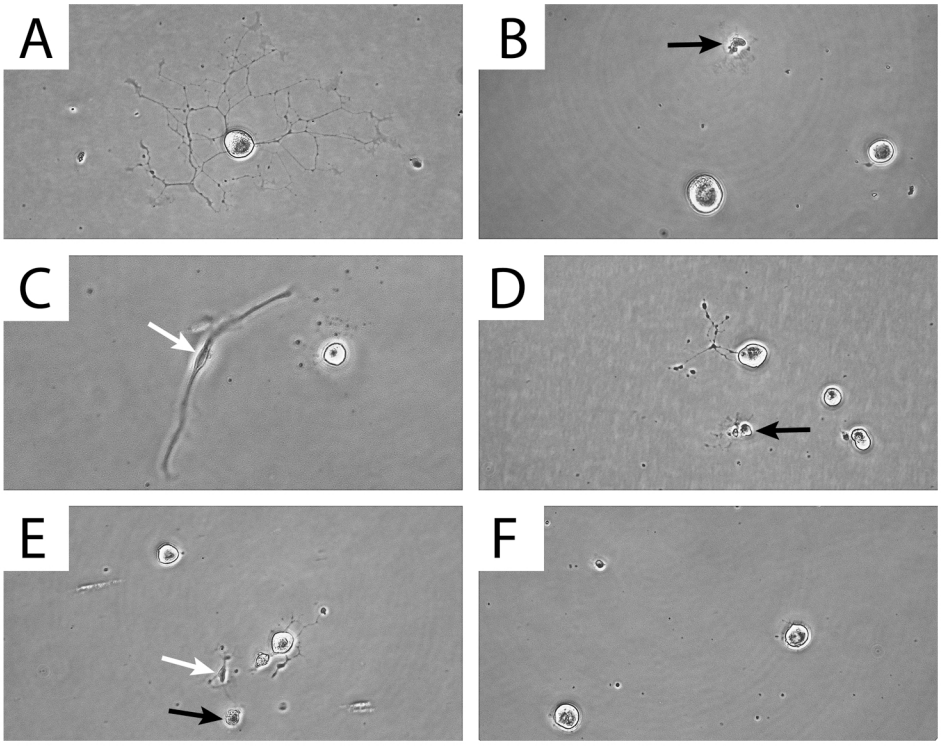
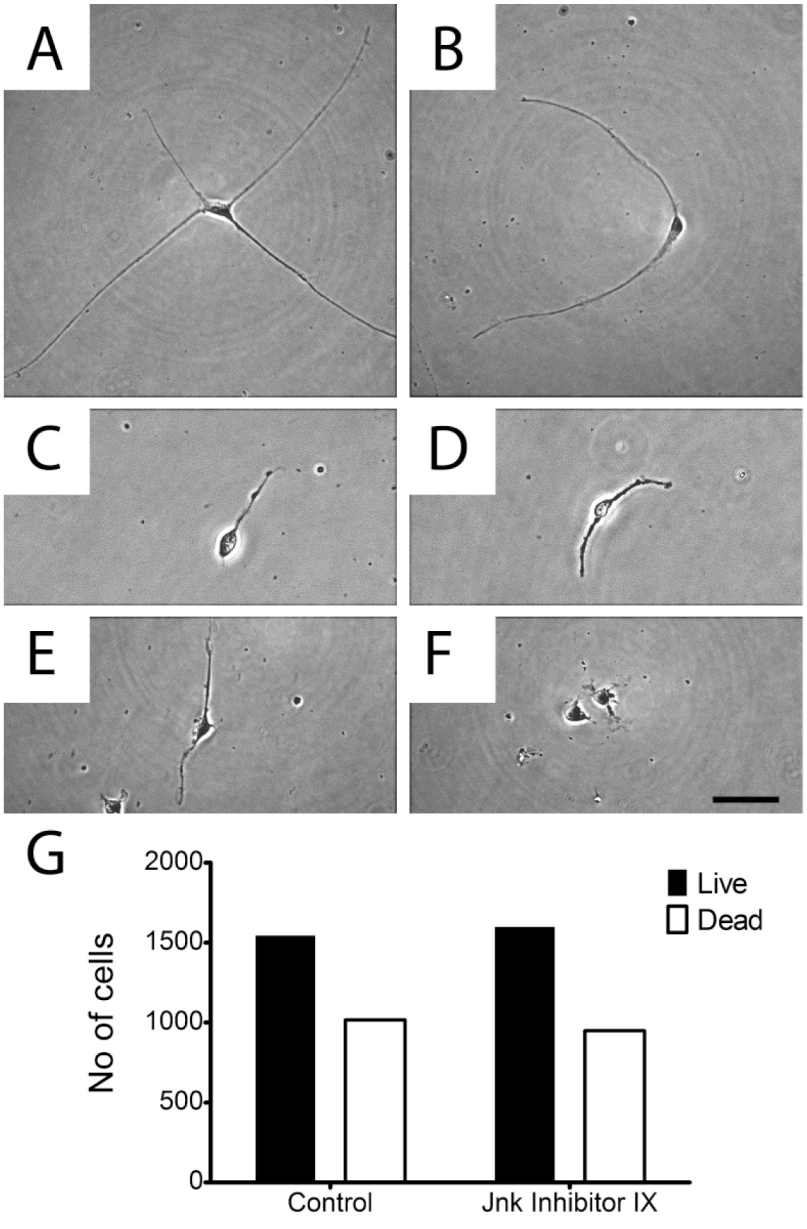


Figure 3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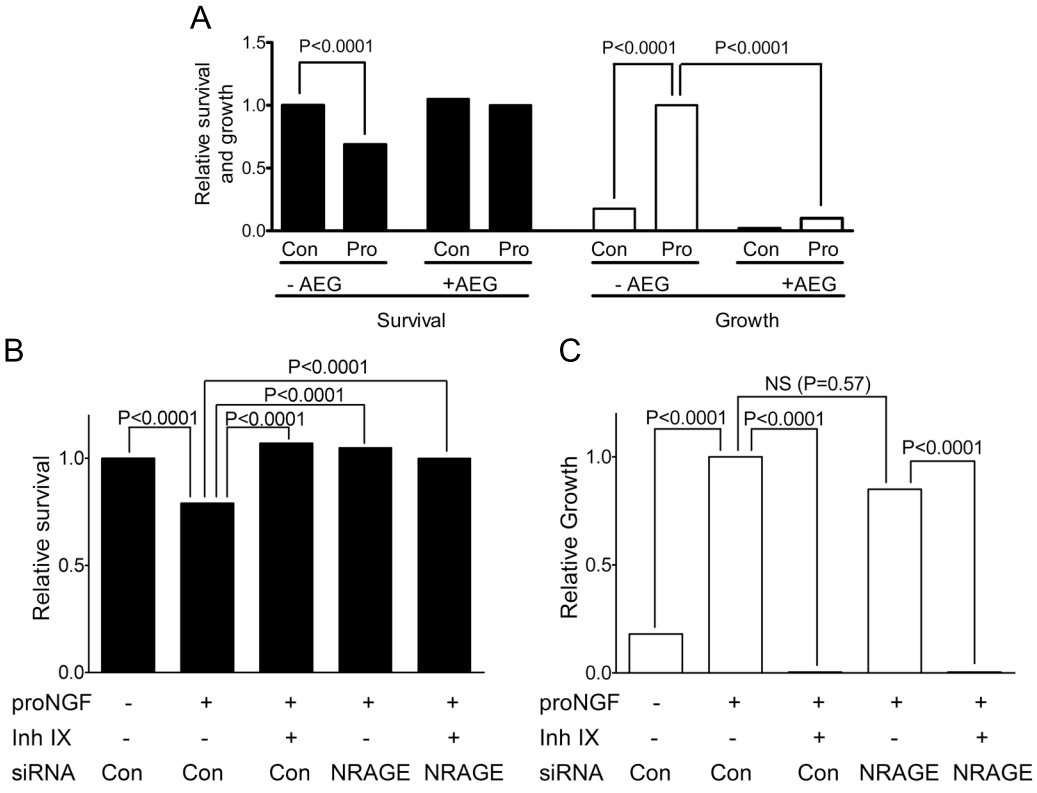
0
No of cells
Live
Dead
Control
Jnk Inhibitor IX

Figure 4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Survival
Growth
B
Relative survival
1.0
0.5
0.0
P<0.0001
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Relative Growth
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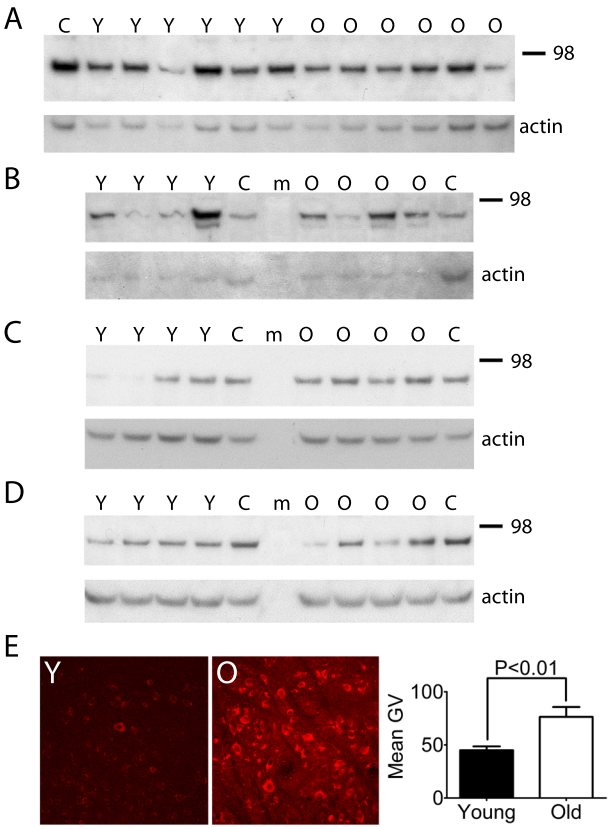
Inh IX - - + - +
siRNA Con Con Con NRAGE NRAGE

Figure 5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Mean GV
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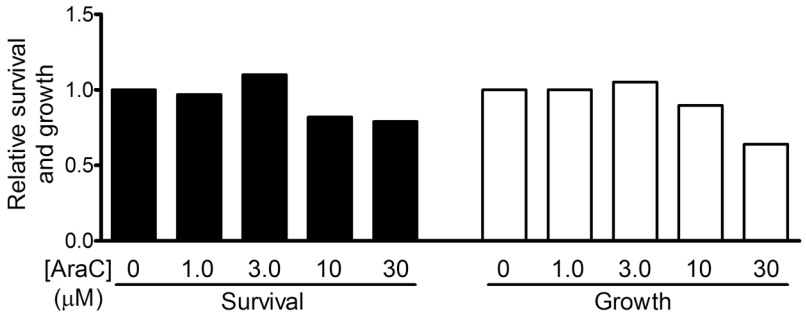
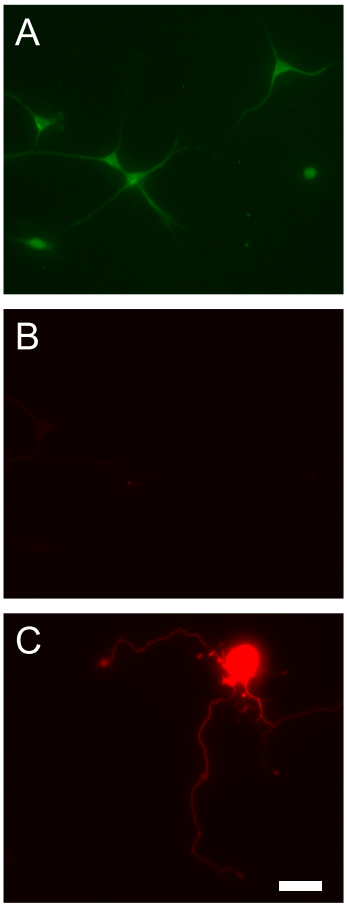
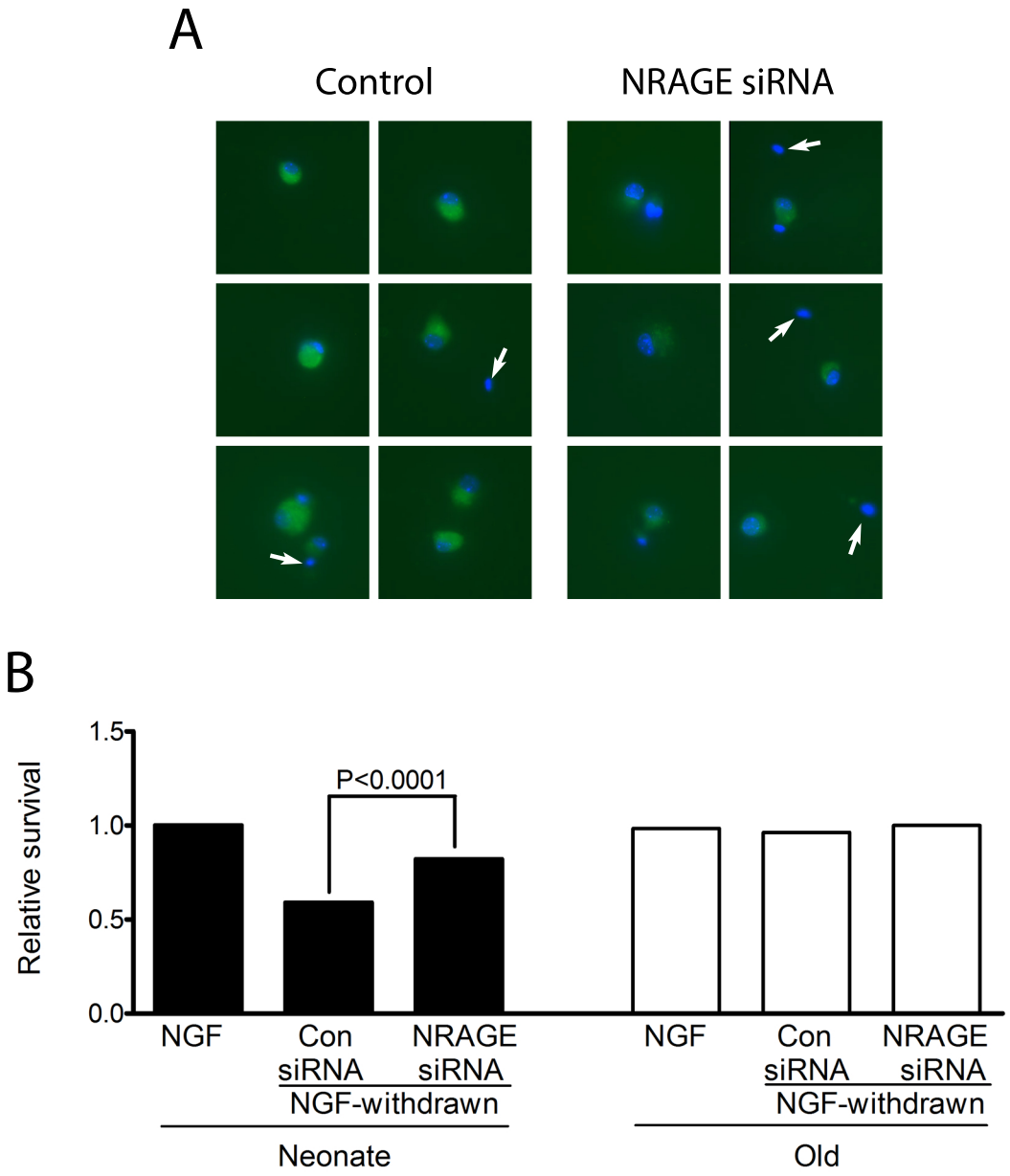
Figure 6



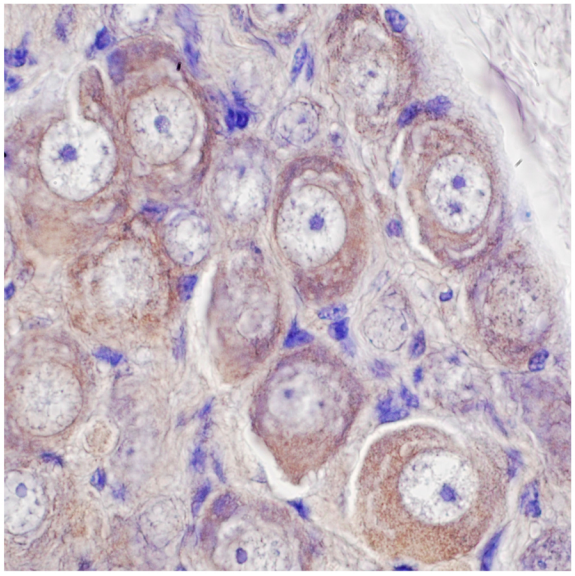
Supplementary Fig. 1. Titration of AraC – effects on survival and growth of aged SCG neurons. Relative survival (filled bars) and growth (open bars) of aged neurons is shown after culture for 48h in the presence of AraC at the concentrations indicated.



Supplementary Fig. 2. Immunofluorescence verification of the identification of glia. (A) & (B): Cells identified on morphological grounds as glia stained positive with anti GFAP (A) and negative with the neuron-specific marker Tuj-1. (C) Tuj-1 stained neuron. Scale bar (C) 50μm.



Supplementary Fig. 3. Validation of NRAGE knockdown.
The effectiveness of NRAGE siRNA treatment was demonstrated directly (A) and by bioassay (B). (A) quantitative reduction of immunofluorescence signal in old-aged neurons treated with penetratin-linked NRAGE siRNA when compared with penetratin-linked control siRNA. Green: NRAGE; blue: nuclei. Arrows point to nuclei of glial cells, which do not express NRAGE. (B) Withdrawal of NGF results in death of SCG neurons isolated from neonates, but not from old-aged animals. Treatment with NRAGE siRNA prevented the death of neonatal SCG neurons that occurs following NGF withdrawal. In contrast, NRAGE siRNA has no effect on the survival of old-aged neurons when compared with control siRNA. n=400 (neonatal neurons); n=900 (old-aged neurons). Threshold P value = 0.0167.



Supplementary Figure 4.

NRAGE expression in the SCG of a 22 month old male mouse. Immunoperoxidase staining of SCG sections with anti-NRAGE shows that the great majority of the immunoreactivity (brown) is confined to neuronal cell bodies. Nuclei are counterstained with hematoxylin (blue).