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ORIGINAL ARTICLE

Neuronal c-Jun is required for successful axonal regeneration, but the effects of phosphorylation of its N-terminus are moderate

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Abstract

Although neural c-Jun is essential for successful peripheral nerve regeneration, the cellular basis of this effect and the impact of c-Jun activation are incompletely understood. In the current study, we explored the effects of neuron-selective c-Jun deletion, substitution of serine 63 and 73 phosphoacceptor sites with non-phosphorylatable alanine, and deletion of Jun N-terminal kinases 1, 2 and 3 in mouse facial nerve regeneration. Removal of the floxed *c-jun* gene in facial motoneurons using cre recombinase under control of a neuron-specific synapsin promoter (junΔS) abolished basal and injury-induced neuronal c-Jun immunoreactivity, as well as most of the molecular responses following facial axotomy. Absence of neuronal Jun reduced the speed of axonal regeneration following crush, and prevented most cut axons from reconnecting to their target, significantly reducing functional recovery. Despite blocking cell death, this was associated with a large number of shrunken neurons. Finally, junΔS mutants also had diminished astrocyte and microglial activation and T-cell influx, suggesting that these non-neuronal responses depend on the release of Jun-dependent signals from neighboring injured motoneurons. The effects of substituting serine 63 and 73 phosphoacceptor sites (junAA), or of global deletion of individual kinases responsible for N-terminal c-Jun phosphorylation were mild. junAA mutants showed decrease in neuronal cell size, a moderate reduction in post-axotomy CD44 levels and slightly increased astrogliosis. Deletion of Jun N-terminal kinase (JNK)1 or JNK3 showed delayed functional recovery; deletion of JNK3 also interfered with T-cell influx, and reduced CD44 levels. Deletion of JNK2 had no effect. Thus, neuronal c-Jun is needed in regeneration, but JNK phosphorylation of the N-terminus mostly appears to not be required for its function.

Keywords: brain repair, peripheral nerve regeneration, phosphorylation, transcription factor.
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Transection of peripheral nerves or their axons (axotomy) usually results in successful regeneration, by a part of the axon still connected to its cell body growing back to and reinnervating the peripheral target (Lieberman 1971; Kreutzberg 1996). Recent studies using transgenic animal models and viral vectors have demonstrated important regeneration-promoting roles for several newly expressed cell adhesion molecules (Werner *et al.* 2000; Hiroi *et al.* 2003, Makwana *et al.* 2009), neurotrophic and growth factor signals (English *et al.* 2011; Makwana *et al.* 2009) and neuropeptides (Sachs *et al.* 2007). The fact that many of these molecular changes occur at the same time following injury has focused interest on axotomy-induced transcription factors that may orchestrate this regenerative response. Deletion of CCAAT enhancer binding protein (cEBP) beta, cEBP delta, sox11 and signal transducer and activator of

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Abbreviations used: CGRP, calcitonin gene related peptide; JNK, Jun N-terminal kinase; PBS, phosphate-buffered saline.

transcription (STAT)3 has been associated with a reduction in the initiation of axonal outgrowth and/or functional recovery (Nadeau *et al.* 2005; Jankowski *et al.* 2009; Magoulas and Lopez De Heredia 2010; Bareyre *et al.* 2011). The effects were particularly pronounced with brain-wide deletion of c-Jun, which prevented the up-regulation of axotomy-associated molecules (CD44, alpha7-beta1 integrin, galanin), decreased successful reconnection to peripheral targets by 4- to 5-fold, and strongly reduced functional recovery (Raivich *et al.* 2004). Interestingly, cre recombinase-mediated deletion of floxed c-Jun was also associated with enhanced neuronal post-axotomy survival, but of severely shrunken motoneurons. Because this cre recombinase was driven by the nestin-promoter, expressed in early neuroepithelial cells giving rise to neurons, astrocytes or oligodendroglia, as well as in Schwann cell precursors, it is still unclear whether the effects observed in neuronal regeneration were due to its absence in neurons or other nestin+ progenitor-derived cells (Rao 1999; Akiyama *et al.* 2001; Kanu *et al.* 2010).

The activator protein 1 (AP1) transcription complex is a well-characterized regulator of neural development and response to trauma, excitotoxic or hypoxic-ischemic injury and consists of homo- or heterodimeric complexes between members of the Jun, c-Fos and activating transcription factor (ATF)/cyclic adenosine monophosphate (AMP) response element-binding protein (CREB) families (Angel and Karin 1992). Its primary component, c-Jun, is produced as an immediate early gene following nerve injury and persists at high levels during the entire peripheral regenerative process (Herdegen *et al.* 1991; Jenkins and Hunt 1991). With the exception of retinal ganglion cell neurons (Herdegen *et al.* 1993; Koistinaho *et al.* 1993), up-regulation of c-Jun is either transient or absent following transection of central fibers, and may contribute to the reduced ability of centrally injured neurons to regenerate (Anderson *et al.* 1998). Activation of the c-Jun mediated transcription apparatus is affected by interactions at three major sites: (i) N-terminal phosphorylation at Ser 63 and 73 and Thr 91 and 93 (Smeal *et al.* 1994; Morton *et al.* 2003) and the ensuing ubiquitination and degradation (Nateri *et al.* 2004), (ii) dephosphorylation of Thr239 (Morton *et al.* 2003) and (iii) the C-terminal lysine acetylation near aa257–276 (Vries *et al.* 2001). The best studied is N-terminal phosphorylation through Jun N-terminal kinases 1, 2 and 3 (JNK1–3), which can alter AP1 binding activity in the absence of de novo protein synthesis (Dérijard *et al.* 1994; Kallunki *et al.* 1994). These JNK mitogen-activated protein kinases are rapidly activated following peripheral nerve injury and increase the amount of phospho-c-Jun in the process (Waetzig *et al.* 2006). Depending on the type of neural injury, deletion of JNK1, 2 and 3 has been shown to a prodegenerative or protective effect (Pirianov *et al.* 2007; Haeusgen *et al.* 2009). Removal of serine 63 and 73 phosphoacceptor sites in the junAA mutant also interferes with exitotoxicity (Behrens *et al.* 1999), but relatively little is known about their involvement in axotomy response and neurite outgrowth *in vivo*. In the current study, we therefore examined the function, cellular basis and possible activation of c-Jun in peripheral regeneration, using mutants with neuron-specific deletion of c-Jun, global deletion of JNK1, 2 or 3, and alanine replacement of the serine 63 and 73 sites.

Materials and methods

Animals, surgical procedures and tissue treatment

Mice carrying floxed *c-jun* allele ($jun^{F/F}$) (Behrens *et al.* 2002) were crossed with syn::cre animals expressing cre recombinase under the control of the synapsin promoter syn::cre (Zhu *et al.* 2001), twice, to obtain homozygous CNS neuron-specific deletion of *jun* (*junΔS*). To prevent germline c-*jun* deletion due to testicular synapsin promoter expression (Hoesche *et al.* 1993; Street *et al.* 2005), only female cre+ mice (syn::cre $jun^{F/F}$) and cre- $jun^{F/F}$ males were used for breeding, generating *jun*ΔS mutants and $jun^{F/F}$ littermate controls with normal Mendelian frequency. Homozygous JunAA, with serine 63 and 73 residues replaced with alanine (A) (Behrens *et al.* 1999) and wild-type (wt) mice were derived as offspring of heterozygous (AA/wt) breeder pairs. JNK1, 2 and 3 null mouse strains (Yang *et al.* 1997; Dong *et al.* 1998; Kuan *et al.* 1999) were crossed with C57Bl/6 mice, to obtain F1 heterozygotes, and in the second step, the F2 homozygote and wild-type breeder pairs that were used to generate mutants and their controls as described in detail elsewhere (Brecht *et al.* 2005).

Mutant and wild-type controls 8–10 weeks old were anesthetised with 10 μg/g 2,2,2-tribromoethanol (Sigma, Dorset, UK), the right facial nerve was either crushed (for the day 4 time point) or cut for all other time points at the stylomastoid foramen, and the animals killed after 4–30 days with an overdose of Euthatal. The animals were first perfused as described by Moller *et al.* (1996) with 150 mL phosphate-buffered saline (PBS) followed by 200 mL of 4% paraformaldehyde in PBS, 2 h post-fixed with 1% paraformaldehyde in PBS, cryoprotected in 30% sucrose in PBS and then frozen on dry ice. The immunohistochemical methods, assessment of axonal regeneration and functional recovery, retrograde tracing and quantification of neuronal survival were performed as previously (Moller *et al.* 1996; Raivich *et al.* 1998, 2002, 2004) and described in detail in the Methods section of the Supplementary material.

Results

Generation of mutant mice

Cell type specific c-jun gene deletion was achieved by crossing mice with a floxed c-jun allele ($jun^{F/F}$) with animals expressing cre recombinase under the control of the synapsin promoter (syn::cre), which targets the *jun*ΔS recombination specifically to neurons (Kügler *et al.* 2001; Zhu *et al.* 2001). Homozygous JunAA and wild-type (wt) mice were derived as offspring of heterozygous (AA/wt) breeder pairs. JNK1, 2 and 3 knockout mice and their controls were produced from F2 homozygous breeder pairs derived from heterozygous

mice on C57Bl/6 background. All mutants used in the current study displayed normal brain structure and histology on H&E and NeuN stains on forebrain (cortex, hippocampus, basal ganglia) and hind-brain (cerebellum, metencephalon) sections (not shown). Homozygous *jun*ΔS recombination was associated with disappearance of basal immunoreactivity throughout the brain, for example in hippocampus and dentate gyrus (Fig. 1a and b), or the uninjured facial motor nucleus (Fig. 1c and d). Motoneuron c-Jun immunoreactivity is strongly up-regulated by facial nerve cut (Fig. 1f) and this increase was abolished in the *jun*ΔS mutant (Fig. 1e). Previous study from our groups revealed the disappearance of phospho-serine63 immunoreactivity in the neuronal nuclei of the axotomized facial motor nucleus in junAA mice but also a persistence of phosphoserine 73-like and as well as of total c-Jun immunoreactivity. In the same study, single JNK deletions were not associated with changes in overall phospho-Jun immunoreactivity (Brecht *et al.* 2005). This persistence of total c-Jun immunoreactivity in neuronal nuclei of the axotomized facial motor nucleus in the junAA mice was also reconfirmed in the current study (data not shown).

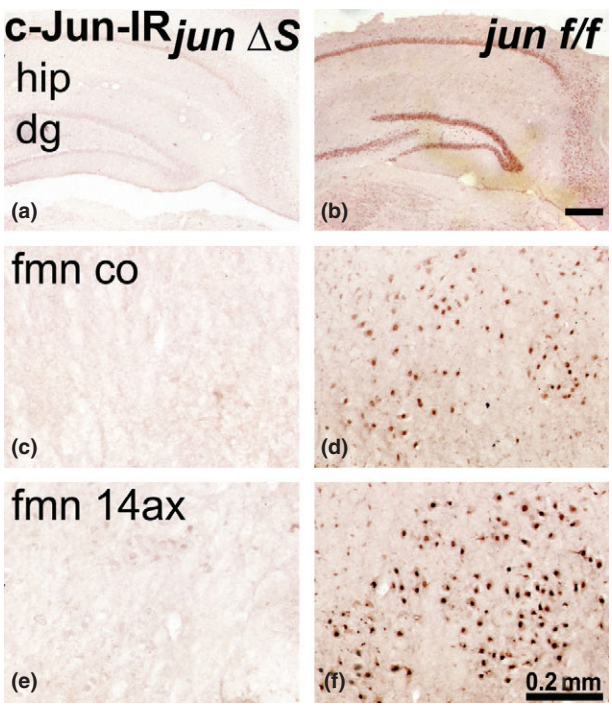

Fig. 1 Synapsin::cre deletion of floxed c-jun (junΔS) removes c-Jun immunoreactivity in normal brain and following facial nerve cut. (a, b) Hippocampus, (c, d) uninjured facial motor nucleus, (e, f) facial nucleus 14 days after facial nerve cut. Micrographs on the left (a, c, e) show the junΔS mutants, those on the right (b, d, f) the control, junF/F animals. Note the strong c-Jun immunoreactivity on the right, in the junF/F hippocampus and hippocampal dentate gyrus (hip and dg), as well as in the large motoneuron nuclei of the facial nucleus (d, f), particularly following nerve injury (f). Scale bar: 0.2 mm.

Deletion of neuronal c-jun interferes with axonal regeneration

Effects on axonal regeneration after facial nerve axotomy at the stylomastoid foramen were assessed by the extent of functional recovery, reinnervation of peripheral target, and speed of axonal elongation in the early phase of nerve regeneration. Functional recovery after facial nerve cut was scored by observed whisker hair movement from 0 (no movement) to 3 (strong, normal movement on uninjured side) by two observers unaware of the animal genotype three times per week for 28 days. As shown in Fig. 2a, $jun^{F/F}$ animals commenced recovery of whisker vibrissae at 12 days and improved steadily over the next 2.5 weeks to day 28. Recovery in homozygous *jun*ΔS mutants was significantly delayed ($p < 0.01$ in unpaired *t*-test, unless indicated, all further tests are also unpaired *t*-test); first noticed at day 19, it then improved but with a less steep trajectory than in the junF/F mice. These differences were mirrored by changes in the regeneration index, calculated as area under the curve for the functional recovery over days 0–28 (Fig. 2b); with an index of 1.00 ± 0.04 for the $jun^{F/F}$ ($n = 5$ animals) and 0.44 ± 0.02 for the *jun*ΔS mutants ($n = 6$ animals, $p < 0.001$).

To determine whether this defect was due to lack of axonal reinnervation or simply reduced function of successfully reinnervated axons, a gelfoam insert soaked with fluorescent tracer Fluorogold (FG) was implanted for 30 min under both whisker pads 28 days after facial nerve cut. The contralateral whisker pad served as an intra-animal, uninjured control. This was followed by 48 h of recovery and retrograde transport to the facial nucleus, and then by perfusion, freezing the brainstem and cutting through the whole brainstem at the level of the facial motor nuclei. The distribution of FG-labelled motoneurons on the the previously uninjured and on the injured side is shown in Fig. 2c,d,g and h. Counting every fifth 20 um section, $jun^{F/F}$ animals ($n = 5$) revealed 168 ± 10 FG+ motoneurons on the uninjured and 57 ± 5 on the injured side. Homozygous *jun*ΔS mutants ($n = 6$) also showed a very similar number (173 ± 9) on the control side, but there was a very drastic reduction on the axotomized side, with just 7 ± 2 FG+ motoneurons (Fig. 1e, $p < 0.001$). In relative terms (as percent of contralateral side) $jun^{F/F}$ animals showed retrograde labelling of 32.9 ±4.4%, and *jun*ΔS mutants a more than 8-fold decrease with 3.8% ± 1.1% (Fig. 1f, $p < 0.001$), exceeding the defect in functional recovery.

As a next step, we explored whether these defects can be attributed to lack of activation of Jun protein function (as opposed to one that is simply absent) by performing these experiments in phosphorylation-deficient homozygous mutants lacking N-terminal phosphoacceptor sites serine-63 and 73 (junAA), or Jun N-terminal Kinases 1, 2 or 3 (JNK1–3), following them for up to 50 days after facial nerve cut. In the case of JunAA (Fig. 3a and b), functional recovery in mutants was on average just barely lower than that in the

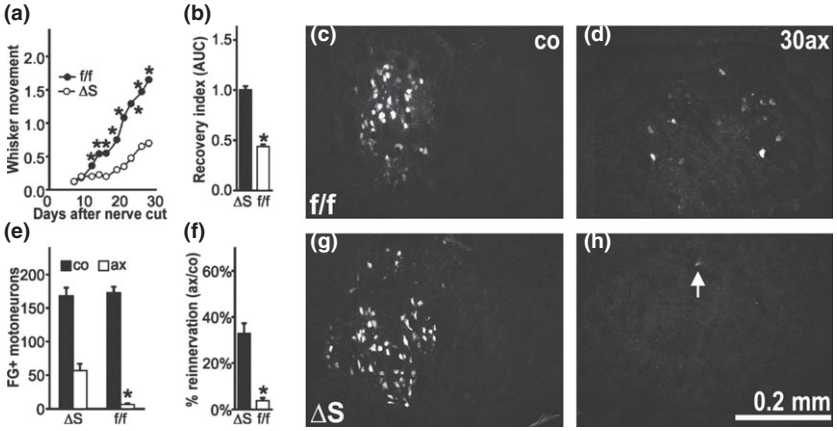

Fig. 2 Neuronal c-Jun is required for successful functional recovery and target reinnervation after facial nerve axotomy. (a, b) Time course for functional recovery of whisker hair movement on the scale from 0 (no movement) to 3 (full movement, equivalent to uninjured side) (a) and the recovery index (b) calculated as area under the curve (AUC) show pronounced reduction and delay in recovery junΔS mice ($n = 6$ mice) compared with the junF/F controls ($n = 5$ mice). *$p < 0.05$ in unpaired *t*-test, bars and error bars in these and all following graphs show the mean and SEM, respectively. In panel (a), the significance levels were adjusted for multiple testing using Bonferroni correction. (c–h) Peripheral target (whisker pad) reinnervation using 48 h retrograde transport of FluoroGold to facial motor nucleus 30 days after nerve cut. (c, d, g, h) Micrographs showing FluoroGold labelling on the contralateral control (co; c, g) and axotomized side (ax; d, h). Retrograde labelling on the uninjured side is confined to a discrete facial subnucleus (c, g); after injury, motoneurons reinnervating the whisker pad are found throughout the facial nucleus (f). (e, f) Number of retrogradely labelled, counted motoneurons on the contralateral and axotomized side (e) and the axotomized/control side ratio (f). Neuronal deletion of c-Jun is associated with a more than 8-fold decrease in whiskerpad reinnervation (c, d and arrow in h).

wild-type controls, with the only significant difference ($p < 0.05$) at 12 days following facial nerve cut. Homozygous deletion of JNK2 (Fig. 3i and j) had no significant effect. In contrast, deletion of JNK1 (Fig. 3e and f) and JNK3 (Fig. 3m and n) showed a significant delay in functional recovery at different time points after nerve cut. Similar effects were also observed for the regeneration index, with significant reduction for JNK1 (Fig. 3f, $p < 0.02$) and a similar trend for JNK3 (Fig. 3n, $p = 0.074$).

To see whether these defects were matched by a deficit in target reinnervation, we next determined the extent of 48 h retrograde transport of Fluorogold, 50 days after facial nerve cut. However, as shown for junAA in Fig. 3c and d, for JNK1 in Fig. 3g and h, for JNK2 in Fig. 3k and l and for JNK3 in Fig. 3o and p, all 4 mutants revealed reconnection rates that were very similar to those for their wild-type controls.

As functional recovery following nerve cut depends on the gradual formation of a non-neuronal cellular bridge between the proximal and distal nerve stump and could be affected by JNK deletions, we also examined the extent of early nerve fibre outgrowth following facial nerve crush, where, despite axotomy, the basal lamina scaffolds remain intact. The growth front of the regenerating motor neurites 4 days after facial nerve crush was quantified in longitudinal facial nerve sections of 10 um thickness using immunoreactivity for the calcitonin gene related peptide (CGRP) and galanin neuropeptides expressed in axotomized facial motoneurons (Werner *et al.* 2000). As shown in Fig. 4a, in the $jun^{F/F}$ control animals' axonal growth front advanced to 5.8 ± 0.1 mm for CGRP- and to 5.4 ± 0.2 mm for the galanin-positive axons. In *jun*ΔS mutants, this axonal elongation was reduced by approx. 60% for both types of neuropeptide containing axons ($p < 0.0001$). In contrast to *jun*ΔS, homozygous deletion of JNK1 (Fig. 4c), JNK2 (Fig. 4d) or JNK3 (Fig. 4e) did not affect the extent of axonal elongation, compared with their wild-type controls. Similar lack of effect on early phase of regeneration following nerve crush was also observed for junAA (Fig. 4b).

Neuronal c-Jun deletion abolishes neuronal cell loss after nerve cut but affected motoneurons show atrophic phenotype

Facial axotomy is normally associated with the cell death of 20–40% of lesioned motoneurons within 30 days after nerve cut (Raivich *et al.* 2002) and pan-CNS deletion of c-Jun has been shown to interfere with this process (Raivich *et al.* 2004). Figure S1a–f shows the effects of neuron-specific deletion of jun on the Nissl stained sections of control (Figure S1a and c) and axotomized (Figure S1b and d–f) facial motor nuclei. After facial nerve cut, $jun^{F/F}$ control animals showed a pronounced loss of neurons (Figure S1b), an effect lost in the *jun*ΔS mutants. However, many of the axotomized *jun*ΔS motoneurons appeared shrunken and with much paler Nissl staining (Figure S1d, compare also Figure S1e and f). Counting motoneuron profiles on the uninjured and injured side throughout the facial motor nucleus revealed a 38 ± 5% loss in $jun^{F/F}$ controls, compared with just 1 ± 4% in the *jun*ΔS mutants (Figure S1i).

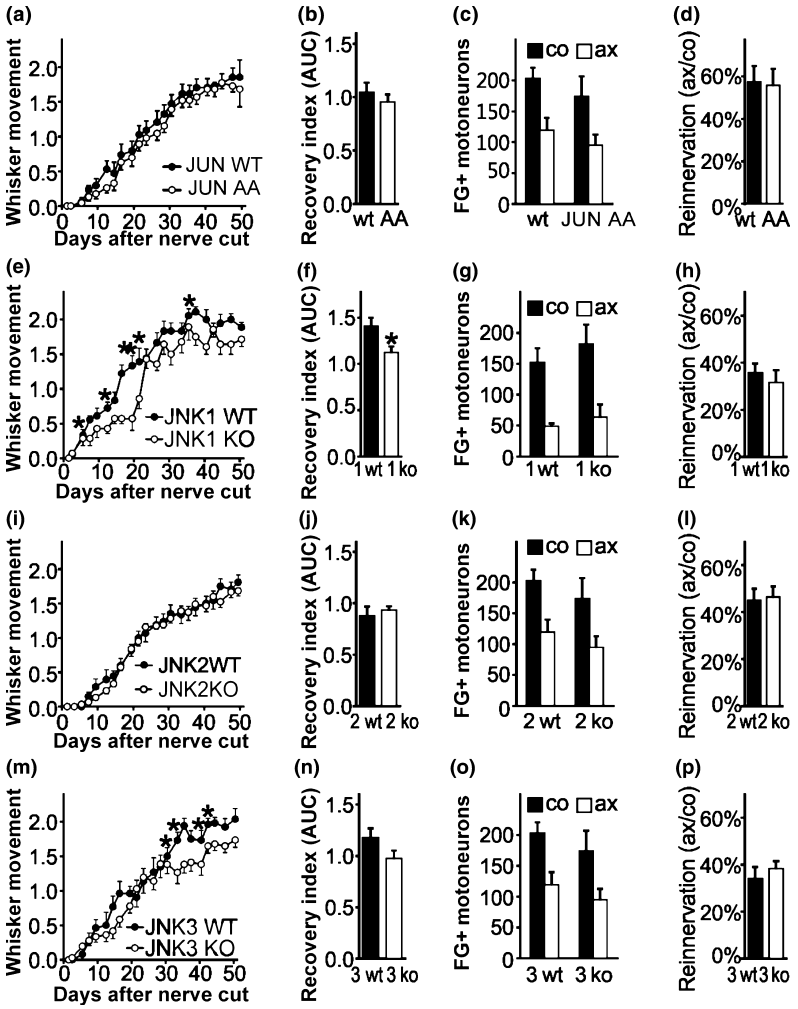

Fig. 3 (a–c, e–g, i–k, m–o) Functional recovery (time course: a, e, i, m; recovery index: b, f, j, n) and whisker pad reinnervation determined by counting retrogradely labelled Fluorogold-fluorescing facial motoneurons (FG+) 30 days after axotomy (c, g, k, o) in JunAA (a–d), JNK1 null (e–h), JNK2 null (i–l) and JNK3 null (m–p) mutants (KO) and their wild-type counterparts (WT). (d, h, l, p) Axotomized (ax)/control (co) side ratio – percent reinnervation – for the FG+ motoneurons. Overall, there was very little effect for junAA (n = 23 mutants and 17 control animals) and JNK2 (n = 30 mutants and 17 controls), but deletion of JNK1 (n = 7 mutants and 9 controls) or JNK3 (n = 18 mutants and 12 controls) was associated with reduced functional recovery (e, f, m). However, none of the four mutants showed an effect on the anatomical reinnervation of the whisker pad at 30 days after facial nerve cut, detected using Fluorogold retrograde labelling (third and fourth column, n = 7/7, 9/10, 6/5 and 6/9 for the junAA, JNK1, JNK2 and JNK3 null mutants and their controls, respectively. *$p < 0.05$ in unpaired t-test. In panels (a, e, i and m), the significance levels were adjusted for multiple testing using Bonferroni correction. Filled bars in the third column (c, g, k and o) show FG+ motoneuron number on the control, contralateral side, and empty bars the axotomized side in the same group of animals. In all other graphs, filled bars or circles represent wild-type, and empty bars, mutant animals.

However, opposite effects were observed on cell size (Figure S1m): in $jun^{F/F}$ controls, average neuronal cell diameter showed a trend towards increase on the axotomized side, from 21.5 ± 0.7 um for the uninjured to 23.2 ± 0.8 um for injured motoneurons ($p = 0.085$), the $jun\Delta S$ mice a decrease from 22.1 ± 0.3 to 15.5 ± 0.8 um ($p < 0.005$). Compared with controls, the junAA mutants exhibited no effect on neuronal loss (33% ± 8% vs. 35% ± 2% for control and mutant mice, see Figure S1j), but still a significant 14 ± 2% decrease in neuronal size 30 days after facial nerve cut ($p < 0.05$, Figure S1n). Deletion of JNK1 or JNK3 was not associated with changes in neuronal loss (Figure S1k and l) or neuronal cell size (Figure S1o and p, respectively).

Neuronal molecular response to injury depends on neuronal c-Jun

In neurons capable of regeneration, axonal injury engenders a switch to a regeneration programme comprising of transcription factor changes, followed by adhesion molecules, neuropeptides and growth factor signalling, and an

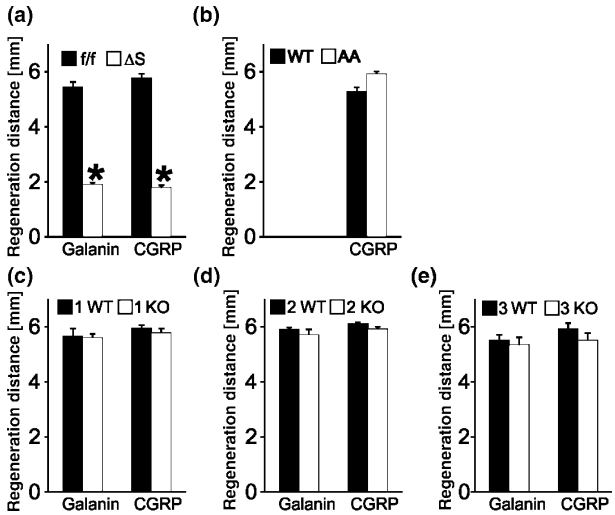

Fig. 4 Facial nerve regeneration relies on the presence of neuronal c-Jun. Longitudinal outgrowth of fastest CGRP- and galanin-immunoreactive axons in mm below the crush site, 4 days after facial nerve injury. (a–e) Effects of junΔS (a, $n = 6$ control junF/F and 4 junΔS animals), junAA (b, $n = 5$ control and 4 mutant mice) and JNK1 (c, $n = 7$ controls and 4 null mutant mice), JNK2 (d, $n = 2$ controls and 3 null mutant mice) and JNK3 (e, $n = 5$ controls and 5 null mutant mice). Note the very similar outgrowth distances for the CGRP- and galanin-immunoreactive axons. JunΔS deletion caused a 65–70% decrease in neurite outgrowth, all other mutations had no significant effect. *$p < 0.05$ in unpaired *t*-test. Only sections stained for CGRP immunoreactivity were available for junAA and its controls.

increase in nutrient transport, metabolism and cytoskeletal protein synthesis (Makwana and Raivich 2005, Raivich and Makwana 2007). As animals for the day 4 time point were used for speed of regeneration experiment in the crushed facial nerve (Fig. 4), the day 4 facial motor nucleus data are following nerve crush; the day 14 data are following cut.

As shown in left upper part of Fig. 5, axotomy of the facial nerve in the $jun^{F/F}$ control animals causes an increase in the immunoreactivity for the adhesion molecules CD44 (Fig. 5a and b) and the beta1 integrin subunit (Fig. 5e and f), neuropeptides CGRP (Fig. 5i and j) and galanin (Fig. 5m and n) and the ATF3 transcription factor (Fig. 5q and r). These axotomy-induced changes are decreased or even, as in the case of CD44, completely abolished in the *jun*ΔS mice (Fig. 5c,d,g,h,k,l,o,p,,s and t). Direct quantification of immunoreactivity in Fig. 6 revealed a significant decrease ($p < 0.05$) in the amount of immunoreactivity on the axotomized side in the *jun*ΔS mouse mutants compared with junF/F controls (Fig. 6a,b,g,h,m,n,s,t,y and ad), as well as a smaller (Fig. 6a,b,h,n,s and y) or even no increase (Fig. 6g,m and t) of the immunoreactivity from the control to the axotomized side, on average, junΔS mice showed an up to 80–100% smaller increase 4 days after facial nerve crush, or 14 days after nerve cut. To determine, whether these changes depend on c-Jun phosphorylation, we next examined changes in CD44, CGRP and ATF3 immunoreactivity in the JNK1, 2 and 3 knockouts and the JunAA mutants at 4 days after nerve crush and 14 days after facial nerve cut. Compared with their controls, JunAA and JNK3 null mutants showed a significant but moderate reduction in CD44 immunoreactivity, at one of the two tested time points – Fig. 6f (−24%), and Fig. 6k (−37%), respectively, for the axotomized to control side difference. The CGRP levels in the case of JNK3 also ATF3 levels were not affected. Deletion of JNK1 (Fig. 6c,i,o,u,z and ae,) or JNK2 (Fig. 6d,j,p,v,aa and af,) had no effect on one of the three tested immunohistochemical neuronal injury-associated markers.

As shown in the Supplementary materials, very similar results – massive inhibition in case of neuronal c-Jun deletion, but at most just moderate effects following deletion of JNK1 or JNK3, or in the junAA mutants were also observed for glial activation and for recruitment of T cells into the facial motor nucleus (Figures S2 and S3).

Discussion

The present data confirm the essential role of neuronal c-Jun for the ability of injured neurons to regenerate following axonal disconnection. Removal of the loxP targeted (floxed) *jun* gene with cre recombinase under the control of a neuron-specific synapsin promoter abolished basal and injury-induced neuronal c-Jun immunoreactivity, as well as most of the molecular response following facial axotomy. This absence of neuronal Jun strongly reduced the speed of axonal regeneration following crush, prevented the vast majority of transected axons after cut from reconnecting to their peripheral target, and impaired functional recovery. Despite blocking neuronal cell death, this was also associated with a large number of shrunken neurons. Finally, junΔS mutants also had diminished astrocyte and microglial activation and T-cell influx, demonstrating that this non-neuronal response depends on the release of Jun-dependent signals from neighboring injured motoneurons.

Compared with complete *jun* gene excision in neurons, the effects of removing serine 63 and 73 phosphoacceptor sites (junAA), or of global deletion of individual kinases responsible for N-terminal c-Jun phosphorylation were mild. The reason global JNK null or junAA mutants were used, because unlike the case with floxed c-Jun, only global mutants were available, and because our primary question was whether the regeneration effects of c-Jun could be reproduced by interfering with its N-terminal phosphorylation. Interestingly, the junAA mutants showed decrease in neuronal cell size, a very moderate reduction in post-axotomy CD44 levels and slightly increased astrogliosis. Deletion of JNK1 or JNK3 showed reduced functional recovery; deletion of JNK3 also interfered with T-cell influx, and somewhat reduced CD44 levels. Deletion of JNK2 had no effect. Thus, neuronal c-Jun is needed in regeneration, but most of this activity may not

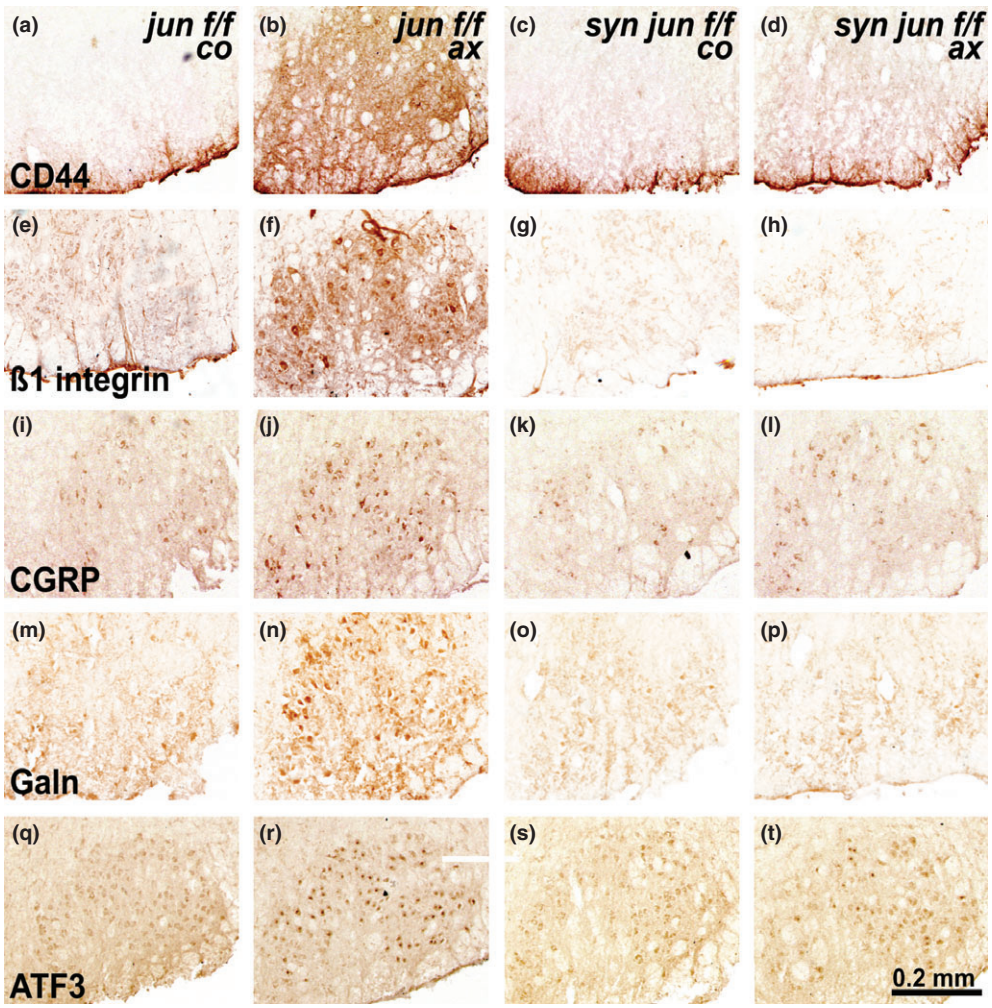

Fig. 5 Biochemical components of neuronal axotomy response depend on the presence of c-Jun. (a–t) Facial nucleus axotomy response 14 days after nerve cut in the junF/F controls (a, b, e, f, i, j, m, n, q, r) and the junΔS mutant (c, d, g, h, k, l, o, p, s, t) mice. Contralateral side (co) -first and third column, Axotomized side (ax) – second and fourth column. After axotomy, junF/F mice show a prominent increase in adhesion molecules CD44 (a–d) and beta1-integrin subunit (e–h), neuropeptides CGRP (i–l) and galanin (m–p) and TF ATF3 (q–t); these changes are reduced or abolished in the junΔS mutants. Scale bar 0.2 mm.

require the presence of individual JNKs or that of the 63 and 73 acceptor sites.

Although cell-type selective excision of target genes is now standard tool in identifying cell-specific function, this can lead to problems if the promoter used to drive cre recombinase is too broad. For example, the nestin promoter used in brain-wide *jun* deletion is expressed in early neuroepithelial cells giving rise to neurons, but also to astrocytes or oligodendroglia. Outside the brain, nestin expression is also found in the islet, liver and muscle stem cells, as well as in Schwann cell precursors (Rao 1999; Akiyama *et al.* 2001; Kanu *et al.* 2010), raising the question whether the regeneration differences observed in that study were also due to *jun* deletion in other cells of neuroepithelial origin. Schwann cells strongly up-regulate c-Jun following axotomy, and removal of Schwann cell c-Jun (Parkinson *et al.* 2008) has recently been shown to interfere with axonal regeneration. This problem is resolved in the present study because synapsin promoter expression is highly restricted, limited to the testis and to maturing and mature neurons in the CNS and peripheral ganglia (Hoesche *et al.* 1993; Street *et al.* 2005).

In the currently used facial axotomy model, deletion of neuronal c-Jun produces four sets of effects: it interferes with the expression of numerous target genes involved in molecular changes after facial nerve axotomy, it impairs the flow of activation and recruitment signals to neighboring non-neuronal cells, it abolishes regenerative capacity in most neurons, and it promotes the survival of shrunken and probably still axotomized neuronal cell bodies. Peripheral nerve cut and crush injuries sometimes elicit differing responses at the level of neuronal cell body (Bareyre *et al.*

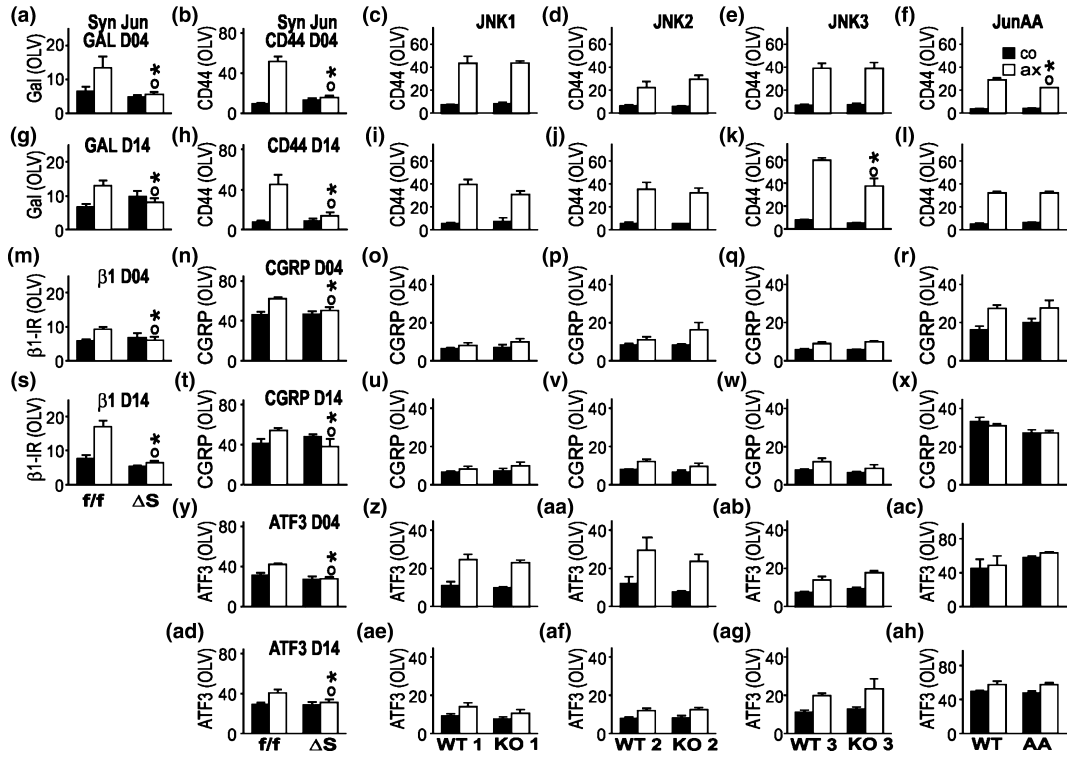

Fig. 6 Effects of neuronal deletion of jun (junΔS, a, b, g, h, m, n, s, t, y, ad, first and second column), the global deletion of JNK1 (c, i, o, u, z, ae, third column), JNK2 (d, j, p, v, aa, af, fourth column), and JNK3 (e, k, q, w, ab, ag, fifth column), and of the junAA mutation (f, l, r, x, ac, ah, sixth column) on changes in neuronal markers following axotomy. Quantification of overall facial motor nucleus immunoreactivity for galanin (a, g) and beta 1 integrin (m, s), CD44 (b–f and h–l), CGRP (n–r and t–x) and ATF3 (y–ah) using OLV units. To facilitate finding the staining and time point on the graph, the row labels (second column, top right of each graph) are the same for the graphs in the third to sixth column). (a–e, m–r, y–ac) 4 days after facial nerve crush, (g–l, s–x, ad–ah) 14 days after facial nerve cut. Filled bars: contralateral (co), empty bars: axotomized (ax) facial nucleus. In each graph, the first two bars represent the data for control animals (wild-type or junF/F) and the third and fourth bar those for the mutant ones. $^*p < 0.05$ (unpaired *t*-test) for the difference on the axotomized side between the mutant and control group. $p < 0.05$ (unpaired *t*-test) for the difference in immunostaining increase due to axotomy (staining intensity on the control side subtracted from that on the axotomized side) between the respective mutant and the wild-type or junF/F control group. $n = 3$–7 animals per group, in each control, and in each mutant group. All five tested markers – galanin, beta1 integrin, CD44, CGRP and ATF3 showed significantly smaller increase or even (g, m, t) the absence of increase, in the junΔS mutants compared with junF/F controls, at both time points – day 4 and day 14. There was no effect for JNK1 or JNK2, and significant but slight decrease for CD44 at day 14 for JNK3 null and at day 4 for junAA.

2011), but in the current case, the molecular responses studied were strongly affected by both types of axotomy, and this response reduced in the absence of c-Jun. Overall, Jun-dependent molecular changes in injured neurons appear pivotal for the latter three sets of functional effects. In the case of axonal regeneration, alpha7beta1 integrin, CD44, galanin and ATF3 have been shown to play an important role in neurite outgrowth. Deletion of the alpha7 integrin gene retards axonal regeneration by 35–40% (Werner *et al.* 2000), removal of the galanin gene delayed sensory functional recovery (Sachs *et al.* 2007); and antibody inhibition of CD44 reduces neurite outgrowth of transplanted central noradrenergic neurons (Nagy *et al.* 1998) as well as creating multiple errors in retinal axonal growth trajectory through the optic chiasm (Lin and Chan 2003). Over-expression of ATF3 also speeds up the initiation of axonal outgrowth, involved in the *in vivo* conditioning response (Seijffers *et al.* 2007). In all four target genes, enhanced expression after injury depends on the presence of neuronal c-Jun. CD44, α7 and galanin have previously described AP1 consensus binding sequences (Lee *et al.* 1993; Nishida *et al.* 1997; Anouar *et al.* 1999) and ATF3 has been identified as a downstream target of c-Jun (Mei *et al.* 2008). Neuronal p53, cathepsinB and noxA have been implicated in axotomy-induced cell death (Kiryu-Seo *et al.* 2005; Sun *et al.* 2010; Tedeschi and Di Giovanni 2009), and axotomy-induced chemokines including fractalkine and monocyte chemotactant protein (MCP)1 (Harrison *et al.* 1998; Flügel *et al.* 2001; Schreiber *et al.* 2001) could contribute to non-neuronal recruitment and activation; here the extent of Jun-dependence for the effects of these signals will need to be demonstrated in future studies.

Previous studies revealed that activation of the c-Jun-mediated transcriptional apparatus is affected by interactions at three major sites: (i) N-Terminal phosphorylation at serine

63 and 73 and threonine 91 and 93 (Smeal *et al.* 1994; Morton *et al.* 2003), (ii) dephosphorylation of threonine 239 involved in the ubiquitination and removal of c-Jun (Morton *et al.* 2003) and (iii) C-terminal lysine acetylation near aa257–276 via p300 (Vries *et al.* 2001; also see Raivich 2008 for a review of the signalling pathways involved in the activation of these Jun-domains). In the case of N-terminal phosphorylation, axonal injury is associated with retrograde transport of phosphorylated JNK, the upstream kinases {[Mitogen-activated protein (MAP)] kinase kinase (MEK or MKK)} kinase (MEKK)1 and phospho - MKK4, as well as the JNK-interacting protein, a scaffold protein that stabilises the interaction between JNK and its upstream kinases (Lindwall and Kanje 2005a,b). Moreover, phosphorylation of Jun will improve dimer stability and thus affect its biological role in the nervous system. Substitution of JNK phosphorylation motifs with those for protein kinase A (PKA) abolishes c-Jun-mediated JNK effects (Smeal *et al.* 1994). Replacement of the serine 63 and 73 phosphoacceptor sites in the junAA mice prevents kainic acid excitotoxity (Behrens *et al.* 1999), to a similar extent as global JNK3 deletion (Yang *et al.* 1997; Brecht *et al.* 2005). Although JNKs have no effect on retinal photoreceptor apoptosis (Grimm *et al.* 2001), junAA and JNK3 null mutants show better neuronal survival after targeted dopaminergic cell death (Brecht *et al.* 2005). Complete JNK ablation – either by deletion or inhibition – has also been shown to decreased caspase-dependent neuronal cell death in both neuronal and non-neuronal cells (Weston and Davis 2007).

Importantly, not all effects of JNK deletion need to be mediated through N-terminal phosphorylation of Jun, and thus would not be observed in the case of junAA – in the current study, this primarily concerns the speed of functional recovery which is normal in junAA, but reduced in JNK1 and 3 null mutations. Interestingly, deletion of JNK3 also strongly reduces cell death of neonatal facial motor neurons after nerve cut, but does not interfere with up-regulation of c-Jun or the phosphorylation of the serine 63 and 73 residues (Keramaris *et al.* 2005). In parentheses, single deletions of JNK2 or 3 significantly decreased cell death in neonatal models (Keramaris *et al.* 2005); as shown in the current study, this effect was not observed following adult axotomy in animals lacking JNK1, 2 or 3 (or the serine 63 and 73 phosphorylation sites), pointing to differences in molecular mechanisms involved in neonatal and adult post-axotomy cell death. So far more than 50 proteins have been shown to be substrates for JNK (Bogoyevitch and Kobe 2006), including various transcription factors in addition to c-Jun (e.g. ATF2, E twenty-six (ETS)-like transcription factor (ELK)1, p53, STAT1/3, etc.), nuclear hormone receptors, apoptosis-regulating bcl2 family members, and signalling and structural molecules involved in cell movement and process outgrowth such as MAP2 (Björkblom *et al.* 2005), MAP1b (Barnat *et al.* 2010) and stathmin (Westerlund *et al.* 2011). Nuclear JNK localization is a requirement for JNK-mediated cell death (Björkblom *et al.* 2008) but effects on neurite outgrowth can be mediated by JNK phosphorylation of cytoplasmic targets (Björkblom *et al.* 2005; Barnat *et al.* 2010; Westerlund *et al.* 2011).

With regards to neurite outgrowth, global *in vitro* inhibition of JNK1/2 and 3 with 10 μM SP600125 strongly reduces and at 200 μM completely blocks outgrowth from the sensory nodose ganglion and dorsal root neurons (Lindwall *et al.* 2004; see also Barnat *et al.* 2010), as well as from sympathetic neurons (Lindwall and Kanje 2005a). *In vitro* studies also showed that deletion of JNK2 or JNK3 interferes with initial dorsal root ganglia (DRG) neurite outgrowth, and deletion of JNK1 and particularly of JNK2 with neurite length. It is possible that combined deletion of all 3 JNKs would have a more pronounced effect, but unfortunately combined deletion of JNK1 and 2 or of all 3 JNKs is embryonic lethal (Kuan *et al.* 1999; Sabapathy *et al.* 1999). Interestingly, functional recovery was not affected in junAA mice (Fig. 3a), suggesting the better performance in the JNK1 and 3 wild-type animals could be due to extra-Jun effects of the JNKs. The regeneration effects shown in the current study in Fig. 3e–p were moderate, with deletions of JNK1 or JNK3 reducing functional recovery, but not interfering with the extent of reinnervation 50 days after facial nerve cut.

In the cerebellar granule cell neurons, inhibition of JNK with 10 uM SP600125, or of the more upstream JNK pathway with mixed lineage kinase inhibitor CEP11004 blocks the ATF3 up-regulation and the ATF3/Phospho-c-Jun mediated cell death following potassium deprivation (Mei *et al.* 2008). However in the current study, deletion of JNK1,2 or 3, or the junAA mutation had no effect on the post-axotomy increase in ATF3 (Fig. 6y–ah), or on neuronal cell death (supplementary Fig. 1). Interestingly, although the junAA mutation did not reduce the cell death it did enhance cell shrinkage. This could suggest that N-terminal phosphorylation brought about through retrograde transport of the kinases (Lindwall and Kanje 2005b) may be important in maintaining the trophic state of surviving neurons. Theoretically, activated JNKs will also lead to the phosphorylation of threonine 91 and 93, which depends on the presence of negatively charged phospho-threonine 95, a target of ataxia teleangiectasia mutated kinase but not of JNK (Vinciguerra *et al.* 2008). Acting in concert, phosphorylation of the three threonines will enhance cell death in response to DNA damage (Vinciguerra *et al.* 2008). Because the three Thr residues are still present in junAA, a similar mechanism may be involved in responsible for reduced functional recovery in the JNK1 or JNK3 null mutants, but not in the junAA mice. Preliminary data using mutant jun4A mice where all 4 JNK-dependent sites were replaced by alanines (S. Patodia, G. Raivich and A. Behrens, unpublished data), do show similar neuronal shrinkage as in junAA, but in addition a moderate but significant decrease in functional recovery (as

with JNK1 or JNK3 deletions) as well as an approximately 40% reduction in whisker pad target reinnervation 30 days after facial nerve cut. Nevertheless, this reinnervation effect is 3–4 times smaller in magnitude than that observed following neuronal deletion of c-Jun (Fig. 2e and f).

It is possible that removal of all 4 phosphoacceptor sites is also needed to reproduce the massive reduction in T-cell influx in the JNK3 null mutants 14 days after facial nerve cut, a peak phase in neuronal cell death, microglial phagocytosis and lymphocyte recruitment (Raivich *et al.* 1998), but this would need to be ascertained in future studies. What the current study does is that it confirms that the pro-regeneration, pro-inflammatory and pro-cell death c-Jun effects observed with nestin-promoter driven expression of Cre recombinase are indeed due to to the neuronal expression of c-Jun. By comparison, the JNK phosphorylation of the N-terminus mostly appears to not be required for these functions.

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Supporting information

Additional supporting information may be found in the online version of this article:

Appendix S1. Supplementary materials and methods.

Figure S1. Deletion of neuronal c-jun induces neuronal shrinkage but prevents cell death.

Figure S2. Neuronal c-Jun is also required for the non-neuronal neuroinflammatory response after facial nerve cut.

Figure S3. Effects of neuronal deletion of c-Jun (junΔS, a, f, k, p, u), global deletion of JNK1 (b, g, l, q, v), JNK2 (c, h, m, r, w), and JNK3 (d, i, n, s, x) and the JunAA mutation (e, j, o, t, y) on microglial and and astrocyte activation and on T-cell recruitment in the facial motor nucleus.

Table S1. Summary of antibody data for the cellular response immunostainings.

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