The AP-1 transcription factor c-Jun is required for efficient axonal regeneration

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Abstract

Nerve injury triggers numerous changes in the injured neurons and surrounding nonneuronal cells that ultimately result in successful target reinnervation or cell death. c-Jun is a component of the heterodimeric AP-1 transcription factor, and c-Jun is highly expressed in response to neuronal trauma. Here we have investigated the role of c-jun during axonal regeneration using mice lacking c-jun in the central nervous system. After transection of the facial nerve, the absence of c-Jun caused severe defects in several aspects of the axonal response, including perineuronal sprouting, lymphocyte recruitment, and microglial activation. c-Jun-deficient motorneurons were atrophic, resistant to axotomy-induced cell death, and showed reduced target muscle reinnervation. Expression of CD44, galanin, and alpha7beta1 integrin, molecules known to be involved in regeneration, was greatly impaired, suggesting a mechanism for c-Jun-mediated axonal growth. Taken together, our results identify c-Jun as an important regulator of axonal regeneration in the injured central nervous system.
The AP-1 Transcription Factor c-Jun Is Required for Efficient Axonal Regeneration

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Summary

Nerve injury triggers numerous changes in the injured neurons and surrounding nonneuronal cells that ultimately result in successful target reinnervation or cell death. c-Jun is a component of the heterodimeric AP-1 transcription factor, and c-Jun is highly expressed in response to neuronal trauma. Here we have investigated the role of c-jun during axonal regeneration using mice lacking c-jun in the central nervous system. After transection of the facial nerve, the absence of c-Jun caused severe defects in several aspects of the axonal response, including perineuronal sprouting, lymphocyte recruitment, and microglial activation. c-Jun-deficient motorneurons were atrophic, resistant to axotomy-induced cell death, and showed reduced target muscle reinnervation. Expression of CD44, galanin, and α7β1 integrin, molecules known to be involved in regeneration, was greatly impaired, suggesting a mechanism for c-Jun-mediated axonal growth. Taken together, our results identify c-Jun as an important regulator of axonal regeneration in the injured central nervous system.

Introduction

The transcription factor AP-1 consists of a variety of dimers composed of members of the Fos and Jun families of proteins (Jochum et al., 2001). While the Fos proteins (c-Fos, FosB, Fra-1, and Fra-2) can only heterodimerize with members of the Jun family, the Jun proteins (c-Jun, JunB, and JunD) can both homodimerize and heterodimerize with other Jun or Fos members to form transcriptionally active complexes (Angel and Karin, 1991; Jochum et al., 2001). In addition to Fos proteins, Jun proteins can also heterodimerize efficiently with other transcription factors, such as members of the ATF/CREB families (Hai et al., 1999). c-Jun is a major component of the AP-1 transcription factor complex and together with JunB and JunD forms the family of mammalian Jun proteins (Mechta-Grigoriou et al., 2001).

The activity of the AP-1 transcription factor is strongly induced in response to numerous signals, including growth factors, cytokines, and extracellular stresses. AP-1 stimulation is mediated, in part, by the phosphorylation of c-Jun by the Jun N-terminal kinases (JNKs) (Davis, 2000). c-Jun N-terminal phosphorylation at the serine residues 63 and 73 and threonine residues 91 and 93 within its transactivation domain is thought to increase transcription of target genes, one of which is the c-jun gene itself (Angel et al., 1988).

c-Jun is expressed during neurogenesis and in the adult brain, but expression is highly induced in response to neuronal injury (Herdegen and Leah, 1998). Nerve fiber transection results in a characteristic reaction of the injured neuron, the so-called axonal response (also termed cell body response or chromatolytic response) (Goldstein, 2001). The axonal response is a sensor and trigger of transcriptional programs of the injured neuron, some of which are mediated by the JNKs. JNKs have been suggested to participate in the initiation of the axonal response because JNKs are rapidly activated following nerve injury and can be transported on microtubules along the axon via their association with motor proteins of the kinesin family (Verhey and Rapoport, 2001). The JNK/c-Jun pathway would therefore be an attractive candidate as a sensor and trigger of the axonal response (Goldstein, 2001).

The goal of the axonal response is to induce and...
support axon elongation and archive a reconstruction of effective synapses. Neurite elongation normally starts at the cut tip of the surviving axon, but additional axonal sprouts can also develop more proximally at the nodes of Ranvier and, occasionally, even at the level of the injured neuronal cell body (Galiano et al., 2001; Linda et al., 1985; Ramon y Cajal, 1928). Failure of axons to reinnervate their target can result in neuronal death, probably due to lack of trophic support of the peripheral (axon) compartment (Goldberg, 2003; Sendtner et al., 1996).

Nerve transection also results in reactive changes in nonneuronal, brain-resident cells of neuroepithelial (astrocytes) and mesodermal/hematopoietic origin (microglia and T cells) (Raivich et al., 1999; Schaiger et al., 1998). In response to axotomy, reactive astrocytes increase the synthesis of cytoskeletal proteins such as GFAP, cytoxines, and extracellular matrix proteins (Eddleston and Mucke, 1993; Fawcett, 1997) and contribute to the formation of the glial scar, which enhances tissue stability but may interfere with any subsequent neural repair or CNS axonal regeneration (McGraw et al., 2001; Pekny et al., 1999). Adjacent microglia, which attach rapidly to injured neurons, are central in the removal of debris from dying cells. Activated microglia appear to play a complex role in neuronal regeneration, by providing trophic factors that mediate survival and axonal outgrowth but also by the synthesis of toxic molecules that may enhance neuronal cell death (Gendelman, 2002; Minagar et al., 2002). Nerve injury also causes a strong recruitment of CD4 and CD8 cell lymphocytes (Bohatschek et al., 2004; Raivich et al., 1998) that appear to enhance neuronal survival (Moalem et al., 1999; Serpe et al., 1999) and the immune surveillance of the injured brain (Maehlen et al., 1989; Raivich et al., 1998).

Transection of the facial nerve is a well-established model system to study the axonal response and neuronal regeneration (Moran and Graeber, 2004). The facial nucleus in the brain stem consists of approximately 1800 motoneurons, which project axons to muscles involved in ear, eyelid, lip, and whisker hair movement (Moran and Graeber, 2004). The facial nerve is bilateral, and therefore the contralateral unoperated side can be used as an intraanimal control.

Although the induction of c-Jun protein by neuronal injury has been well described, the precise role of c-Jun in response to axonal damage is controversial, as studies have linked c-Jun to both axonal regeneration and axotomy-induced cell death (Herdegen et al., 1997). Whereas c-Jun expression did not correlate with neuronal death after fimbria fornix transection (Butterworth and Dragonow, 1996), overexpression of a c-Jun dominant-negative protein inhibited axotomy-induced dopamine neuron death (Crocker et al., 2001). Whether c-Jun is only a marker of the transcriptional regeneration program or required for successful axonal regeneration was unclear, in part due to the embryonic lethality of c-jun-deficient mice (Hilberg et al., 1993; Johnson et al., 1993).

To clarify the role of c-Jun in neuronal development and regeneration, we have used conditional mutagenesis to generate mice lacking c-Jun in neural cells (c-jun−/− mice) (Behrens et al., 2002, 2003). In this study, we show that CNS-specific inactivation of c-jun causes only minor defects in neurogenesis, since the only developmental phenotype we could detect was an increase in the number of facial motoneurons. Moreover, c-Jun appears to be dispensable for hippocampus-dependent learning behavior and amygdala-dependent fear-conditioning responses. However, the absence of c-Jun led to a severe defect of the axonal response, culminating in impaired target reinnervation.

Results

Generation of Mice Lacking c-jun in the CNS
To define the role of c-jun in the axonal response following neuronal injury, we created animals with a nervous system-specific deletion of the c-jun gene by crossing mice carrying a floxed c-jun allele (c-junf/f) with animals expressing cre recombinase under the control of the nestin promoter, which causes efficient recombination in the central and peripheral nervous system (Behrens et al., 2002; Tronche et al., 1999). Previous studies have demonstrated the efficiency and CNS specificity of the nestin-cre transgenic line (Gass et al., 2000; Knoepfler et al., 2002; Mantamadiotis et al., 2002; Tronche et al., 1999). However, Cre/LoxP-mediated recombination frequencies are influenced by the chromosomal location of the recombination substrates and marked differences in recombination efficiency between different floxed genes have been observed (Vooijs et al., 2001). To exclude compensatory mechanisms potentially resulting from homozygous c-jun−/− inactivation, recombination was first investigated in a heterozygous c-jun+/−; Nestin-cre+ mouse. Southern blot analysis revealed efficient c-jun− inactivation in brain and partial deletion in kidney and testis, but c-jun− recombination could not be detected in several other tissues, including thymus and spleen (Figures 1A and 1B). Significant c-jun− inactivation was not confined to a specific brain region, as it was observed in cerebellum (Cb), cortex (Cx), brain stem (Bs), and hippocampus (Hc) (Figure 1C). In contrast, c-jun− recombination was not detectable in FACS-sorted T cells (CD3 positive) and macrophages (CD11b positive) isolated from the spleen of a c-jun−/−; Nestin-cre+ mouse, further confirming the absence of Nestin-cre-mediated recombination in lymphoid and myeloid cells (Figure 1C).

Mice lacking c-jun− in neural cells (c-jun−/−) were born with Mendelian frequency and were viable and fertile. c-jun−/− mice lacked neuronal c-Jun immunoreactivity but showed normal brain histology (Figures 2A–2D) and normal expression of neural markers such as NeuN, MAP-2, and Tuj-1 (data not shown). Whereas the overall architecture and histology of c-jun− brains appeared normal, neuronal cell counts of the facial nucleus revealed a 19% increase in facial motoneurons, with 1716 ± 68 in control c-jun+/+ mice compared to 2038 ± 101 in c-jun−/− mice (p < 5% in unpaired Student’s t test [uSTT]). However, there was no significant change in the number of hypoglossal neurons or L5 DRG neurons, with 793 ± 32 and 838 ± 44 for the c-jun+/+ and c-jun−/− hypoglossal neurons and 2324 ± 266 and 2308 ± 265 for the DRG neurons, respectively.

Moreover, the morphology of the sciatic nerve was similar in mutant and control animals as was the motor performance in a rotating rod assay (data not shown).
Figure 2. Normal Hippocampal Morphology and Behavior in c-jun<sup>−/−</sup> Mice

(A and B) Histological analysis (hematoxylin and eosin staining [H&E]) of hippocampal sections of control animals (c-jun<sup>f/f</sup>) and c-jun<sup>Δn</sup> mice. (C and D) c-Jun immunostaining on hippocampal sections of c-jun<sup>f/f</sup> and c-jun<sup>Δn</sup> mice. (E and F) Immunohistochemistry for Neuronal Nuclei (NeuN) on hippocampal sections of c-jun<sup>f/f</sup> and c-jun<sup>Δn</sup> mice.

In addition, no significant differences between c-jun<sup>f/f</sup> and control mice in hippocampus-dependent learning behavior and amygdala-dependent fear-conditioning responses were observed (Figures 2G–2J), suggesting that the establishment and function of the neuronal cir-
axon elongation and axonal sprouting. To investigate the subsequent lack of trophic support (Sendtner et al., 2000), we performed experiments using a model of Broca after transection of the fimbria fornix, in the axonotome system. Basal c-Jun protein levels were detected in un-axotomized facial motoneurons (Figures 3A and 3E), compared to 20.6% in control mice. After axotomy showed that 62.0% of motoneurons had reconnected to the whisker pad, compared to 20.6% in control animals. Western blot analysis confirmed that the absence of c-jun in c-jun−/− mice was due to differences in muscle reinnervation.

To define the kinetics of axonal regeneration, a second experiment we determined the recovery of whisker movement after 15 weeks as a measure of the animals’ ability to recover normal function. Control mice (Figures 3C, 3D, 3G, 3H) showed strong recovery, whereas c-jun−/− mice (Figures 3A, 3B, 3F, 3I) showed a significant delay in functional recovery but did not abolish all axonal regeneration. Overall, recovery was 62.0% ± 7.3% of control animals. c-jun−/− motoneurons had reconnected to the whisker pad, compared to 20.6% ± 4.9% of control animals (Figures 4B, p < 0.01%). The absence of c-jun in c-jun−/− mice did not abolish all axonal regeneration.

c-jun-Deficiency also interfered with axonal sprouting. Facial axotomy normally leads to enhanced axonal sprouting (Supplemental Figures S2B and S2F at http://www.neuron.org/cgi/content/full/43/1/57/DC1), with a maximum at day 14 (Galiano et al., 2001; Werner et al., 2000), which is absent on the contralateral side (Supplemental Figures S2A, S2C, S2E, and S2G). Control, c-jun+/+ animals showed the prominent sprouting of galanin- (Supplemental Figure S2B, arrows) and CGRP-immunoreactive axons (Supplemental Figure S2F, arrows), but c-jun deficiency interfered with perineuronal sprouting of both types of axons (Supplemental Figures S2D and S2H). Absence of c-jun reduces axotomy-induced cell death but causes neuronal atrophy.

A substantial number of neurons die after nerve transection, probably due to failed target reinnervation and subsequent lack of trophic support (Sendtner et al., 2000), followed by a 72 hr period of retrograde transport (Figures 4C–4F). Fluorogold injection on the unjured side served as intraanimal control, with similar numbers of retrogradely labeled motoneurons per 20 μm section, 22.9 ± 3.4 (n = 4) and 26.6 ± 5.1 (n = 3) on the unjured side in control and mutant mice, respectively. The overall ratio of labeled neurons on the axotomized versus the contralateral side (ax/co ratio) in control animals was 22.4% ± 8.0%. In the absence of c-jun, it was 5.2% ± 1.1%, more than 4-fold lower (Figure 4B, p < 0.01%). The absence of whisker movement in c-jun−/− mice is due to insufficient reinnervation.

To determine if the defective whisker movement of c-jun−/− mice was due to differences in muscle reinnervation, the fluorescent tracer Fluorogold was injected into the whisker pad 28 days after the facial nerve was cut (Werner et al., 2000), followed by a 72 hr period of retrograde transport (Figures 4C–4F). Fluorogold injection on the unjured side served as intraanimal control, with similar numbers of retrogradely labeled motoneurons per 20 μm section, 22.9 ± 3.4 (n = 4) and 26.6 ± 5.1 (n = 3) on the unjured side in control and mutant mice, respectively. The overall ratio of labeled neurons on the axotomized versus the contralateral side (ax/co ratio) in control animals was 22.4% ± 8.0%. In the absence of c-jun, it was 5.2% ± 1.1%, more than 4-fold lower (Figure 4B, p < 0.01%). The absence of whisker movement in c-jun−/− mice is due to insufficient reinnervation.
c-jun Function in Axonal Regeneration

Figure 4. c-Jun Is Required for Efficient Target Reinnervation
(A) Posttraumatic whisker hair motor performance was measured 28 days after facial nerve cut, on a scale of 0 (no movement) to 3 (normal strong movement as on the unoperated side); n = 6 for c-jun−/− and n = 7 for c-jun+/- animals. *p < 1% between c-jun+/- and c-jun−/− groups in unpaired Student’s t test (uSST).
(B) Quantification of retrograde labeling of facial motoneurons with fluorogold (FG), performed 28 and 103 days after facial nerve cut. Neurons in the injured facial motor nucleus of control day 4 (Figure 6A), but late activation measured 14 days after facial nerve cut. (C–F) Fluorogold fluorescence in the facial nucleus in c-jun−/− mice, white bars. *p < 1% between c-jun−/− and c-jun+/- groups. (C–F) Fluorogold fluorescence in the facial nucleus in c-jun−/− and c-jun+/- mice on the control (co) and operated side (ax) 28 days after facial nerve cut. Note the shrinkage of axotomized c-jun+/- neurons ([F], arrows). Scale bar, 100 μm in (C–F).

Figure 5. Decreased Cell Death and Atrophy of Axotomized c-jun−/− Motoneurons
(A) Quantification of motoneuron number in the facial nucleus in c-jun+/- and c-jun−/− mice on the control (white bars) and operated side (black bars), 31 days after facial nerve cut. *p < 0.1% in uSST between c-jun−/− and c-jun+/- groups (A and B), n = 8 for c-jun−/− and n = 7 for c-jun+/- animals in (A) and (B). (B) Quantification of motoneuron cell death (ncd) in the facial nucleus in c-jun−/− and c-jun+/- mice on the control (white bars) and operated side (black bars), 31 days after facial nerve cut. (C–F) Nissl staining in the facial nucleus in c-jun−/− and c-jun+/- mice on the control (co) and operated side (ax), 31 days after facial nerve cut. Note the shrinkage of axotomized c-jun+/- neurons ([F], arrows). Scale bar, 100 μm in (C–F).

1996). To assess whether c-Jun plays a role in axotomy-induced cell death, all motoneurons in the facial motor nucleus were counted using 25 μm cresyl violet-stained serial brain stem tissue sections. One month after facial nerve transection, axotomy had caused a loss of 32% of neurons in the injured facial motor nucleus of control mice, 1165 ± 84 compared to 1716 ± 68 on the unoperated side (n = 8) (Figure 5A). In c-jun+/- mice, neuronal loss by axotomy was reduced to 9% (1829 ± 60 motoneurons on the axotomized and 2038 ± 101 on the unoperated side, p < 0.1% in uSST) (Figure 5B). Nissl staining revealed that facial mutant but not c-jun+/- motoneurons had an atrophic morphology after axotomy (Figures 5C–5F). Quantification showed a reduction in mean cell diameter of axotomized mutant neurons from 18.92 ± 0.41 to 16.08 ± 0.34 μm (co and ax side, respectively), compared with no change in the c-jun+/- animals (18.52 ± 0.18 and 18.62 ± 0.18 μm). These data show that in response to axotomy c-jun is required to execute facial motoneuron cell death and to prevent neuronal atrophy.

Defective Activation of Nonneuronal Cells without c-jun
Nerve transection does not only trigger changes in the neuron itself, but also results in reactive changes in nonneuronal, brain-resident cells of neuroepithelial (astrocytes) and hematopoietic (microglia and T cells) origin (Ravich et al., 1999; Schwaiger et al., 1998). In c-jun+/- mice, early microglial activation, as measured by the increase in immunoreactivity for the αM integrin subunit (also known as MAC-1), was mildly impaired at day 4 (Figure 6A), but late activation measured 14 days after axotomy was strongly diminished compared to...
Figure 6. Reduced Activation of Nonneuronal Cells by Axotomy without c-Jun

(A and B) Quantification of microglial \( \alpha M \) immunoreactivity at day 4 (d4) (A) and day 14 (B) after axotomy using the MEAN-SD algorithm (Moller et al., 1996). Empty bars, contralateral; filled bars, axotomized side. *p < 0.1% in uSST between c-jun\(^{ff} \) and c-jun\(^{Δn} \) groups (A–D). n \( = 5 \) for c-jun\(^{ff} \) and n \( = 6 \) for c-jun\(^{Δn} \) animals in (A); n \( = 8 \) for c-jun\(^{ff} \) and n \( = 7 \) in (B)–(D). (C) Quantification of astrocytic GFAP immunoreactivity at day 14 (d14). (D) CD3-positive T cells in the axotomized facial motor nucleus, day 14. (E–H) \( \alpha M \) immunoreactivity in the facial nucleus in c-jun\(^{ff} \) and c-jun\(^{Δn} \) mice on the control (co) and operated side (ax). (I–L) Double immunofluorescence for c-Jun (red)/\( \alpha M \) (green) (M) and for c-Jun (red)/CD3 (green) (N) in the axotomized facial nucleus of c-jun\(^{ff} \) mice, 14 days after axotomy. (O and P) Double immunofluorescence for c-Jun (red)/\( \alpha M \) (green) (O) and for c-Jun (red)/CD3 (green) (P) in the axotomized facial nucleus of c-jun\(^{Δn} \) mice, 14 days after axotomy. Scale bar, 400 \( \mu m \) in (E)–(L), 50 \( \mu m \) in (M)–(P).

c-jun\(^{ff} \) animals (~88%; Figures 6B and 6E–6H). Axotomized facial motor nuclei in c-jun\(^{ff} \)-deficient brains were also devoid of microglial nodules (Figure 6H), a highly consistent response to neuronal debris 14 days after facial nerve cut in control mice (Figure 6F, arrows) (Raiovich et al., 1999; Streit, 2000), suggesting that the absence of late microglial activation is a result of reduced axotomy-induced neuronal death in c-jun\(^{ff} \) mice (Figures 5A and 5B). Astroglial activation measured by GFAP immunoreactivity was also impaired in c-jun\(^{ff} \) mice, but showed a more moderate reduction (Figures 6C and 6I–6L). c-Jun function was also essential for T cell recruitment in response to facial nerve injury, since at day 14, at the peak of lymphocyte influx into the axotomized facial motor nucleus (Raiovich et al., 1998), the number of CD3-positive T cells was reduced by 92% (Figure 6D).

As shown in Figure 1C, the nestin promoter was not active in CD3+ lymphocytes or \( \alpha M \)-positive macrophages. Moreover, c-Jun immunoreactivity was not detected in the CD3-positive infiltrating cells or in local \( \alpha M \)-positive activated microglia in the axotomized facial motor nucleus in c-jun\(^{ff} \) as well as in c-jun\(^{Δn} \) animals (Figures 7M–7P), indicating that the impairment of microglial activation and T cell recruitment is secondary to a function of c-Jun in neurons.

c-jun Is Required for Expression of Regeneration-Induced Molecules

In order to understand the molecular mechanism of c-Jun function during the chromatolytic response, we explored whether the expression of molecules associated with regeneration was dependent on c-Jun. Since previous studies pointed to an important role of the neuropeptide galanin, the neuronal adhesion molecules \( \alpha 7 \beta 1 \) integrin, and the CD44 hyaluronic acid receptor in neurite elongation (Jones et al., 1997; Jones et al., 2000; Lin and Chan, 2003; Werner et al., 2000; Wynick et al., 2001), we determined whether they were downstream targets of c-Jun action. As demonstrated in Figures 7A–7L, all three molecules show a strong increase in protein expression on the axotomized side in control c-jun\(^{ff} \) animals, a response severely compromised in the brains...
of c-jun-deficient mice. Quantification revealed that axotomy-induced expression was 92% lower for CD44 (Figure 7M), 74% lower for galanin (Figure 7N), and 84% lower for α7b1 integrin (Figure 7O) in c-jun-hf animals compared to control c-jun-hf mice. The induction of CD44 expression was also dependent on the presence of c-Jun in four other types of injured CNS neurons (Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/43/1/57/DC1), underscoring the importance of c-Jun in the induction of axonal growth-associated molecules after nerve injury.

Discussion

Increased synthesis and activity of neuronal transcription factors such as c-Jun, ATF3, and STAT-3 are among the most consistent changes in injured central and peripheral neurons and have been suggested to play an important role in the neural response to injury and the initiation of the neuronal repair program (Herdegen and Leah, 1998; Schweizer et al., 2002; Tsujino et al., 2000). In the current study, we have used CNS-specific inactivation of c-jun to demonstrate that the c-Jun transcription factor is an important regulator of the neuronal response to axonal disconnection. However, we would like to emphasize that, although neurogenesis appeared to be unaffected by the absence of c-jun, we cannot formally exclude the possibility that the regeneration failure in c-jun-hf animals is caused by c-jun deficiency during development.

Increased Facial Motoneuron Number in c-jun-hf Mice

Neuronal development of c-jun-hf animals was largely normal. CNS and peripheral nerve morphology did not reveal any gross abnormalities, and the animals showed normal learning and fear conditioning and unimpaired motor performance. However, c-jun deficiency led to an increase in the number of facial motoneurons, although it did not affect the abundance of DRG sensory neurons and hypoglossal neuron number. It is possible that the increase in the number of facial motoneurons is due to a decrease in cell death during embryonic development, in agreement with previous reports showing reduced death following c-jun-inactivation (Palmada et al., 2002). In the view of the specific control of motoneuron number by c-jun, particularly after injury, it will be interesting to investigate whether c-jun is also involved in motoneuron death caused by pathological conditions such as amyotrophic lateral sclerosis (ALS).

Cell Type Specificity of c-Jun Function during the Axonal Response

The axonal response is a complex genetic program that involves not only neurons but several other cells types of neural and nonneural origin. The axonal response to facial nerve cut in c-jun-hf mice was accompanied by a mild reduction in astrogliosis and an almost complete disappearance of T lymphocyte influx and microglial activation in the axotomized facial motor nucleus, the latter two cell types (T cells, microglia) being of mesodermal/hematopoietic and not of neuroepithelial origin.

As we observed impaired responses to axotomy in both neural and nonneural cells in c-jun-hf mice, for the interpretation of our data it is critical to know whether c-Jun function is required primarily in neurons or whether c-Jun controls axonal regeneration by acting also in other (hematopoietic) cell types. This depends on the neural specificity of the nestin-cre line that was used to inactivate c-jun. Previous reports showed that this line provides efficient recombination activity in the CNS, which was confirmed in this study. However, the deletion frequencies in T cells and microglia/macrophages were not known.

T cells are strongly recruited from the periphery to the damaged brain, particularly in mouse injury models as well as in some human neurological conditions, including ALS (Engelhardt et al., 1993; Kawamata et al., 1992; Raivich et al., 1998). As shown in Figure 1, nestin promoter-driven expression of cre recombinase does not lead to the excision of floxed c-jun in thymus and spleen nor in sorted CD3+ T cells. Thus, the lack of T cell recruitment to the injured facial nucleus is probably a consequence of the absence of c-jun in neural cells. c-Jun may be required for the production of molecules that are involved in T cell chemotaxis. Alternatively, impaired T cell recruitment could also be caused by the reduction of motoneuronal cell death, which may release signals triggering T cell recruitment. Interestingly, mouse mutants lacking T cells, particularly the CD4+ lymphocyte subset, appear to suffer from enhanced motoneuron cell death after axonal injury (Bohatschek et al., 2004; Serpe et al., 1999). This is in marked contrast to the reduced cell death seen in c-jun-hf mice. Hence, the lack of T cell recruitment cannot explain the c-jun mutant phenotype.

The Role of c-Jun in Axotomy-Induced Microglial Activation

In the current study, cre recombinase activity was also not observed in macrophages of c-jun-hf mice (Figure 1C). Microglia and macrophages share a common ontogenic origin, and some microglia may develop from adult migratory peripheral macrophages (Bechmann et al., 2001; Flugel et al., 2001). In addition, reconstitution experiments using genetically marked bone marrow showed that after axotomy a significant percentage of activated microglia was derived from peripheral hematopoietic cells that entered the CNS and differentiated into microglia in situ (Priller et al., 2001). By extension, this speaks against nestin-cre activity in the microglia, although it may not permit us to formally rule out c-jun inactivation in a specific microglial subpopulation. However, the αM-positive microglial cells do not show any nuclear c-Jun immunoreactivity (Figures 6M and 6O), arguing against the involvement of microglial c-Jun in the activation of this brain-resident, macrophage-related cell type. As in the case for T cells, we currently favor the interpretation that the defects in microglial activation observed 14 days after facial nerve cut are caused by the survival of facial motoneurons in c-jun-hf mice. In agreement with this notion, there is only a slight reduction of microglial activation in c-jun-hf mice during the early phase of the axonal response before the onset of neuronal cell death, whereas the drastic difference of microglial activation at a later stage coincides with the peak of cell death at day 14 (Figures 6A and 6B).
Assessment of Target Reinnervation

To functionally evaluate the role of c-jun in axon growth, we employed two assays: retrograde labeling and whisker performance. Retrograde labeling showed a striking and persistent decrease in the ability of c-jun−/− motoneurons to reinnervate the whisker pad, with a similar 3- to 4-fold reduction at 4 and 15 weeks compared to c-jun+/+ controls (Figure 4). Significant differences were also present in motor performance. However, these differences were more significant early after axotomy and not as pronounced at later time points. The assessment scale of whisker movement appears to be nonlinear, because reinnervation of the first 20% of neurons (after 1 month in control mice) resulted in an increase in whisker performance from 0 to 1.7, but an increase from 20% to 62% reinnervation between 4 and 15 weeks only resulted in a comparatively small augmentation to 2.4 (Figure 4). Thus, whisker hair movement performance appears to be a good indicator for the reestablishment of functional connections by the first 20%–30% of the reinnervating neurons, but progressively loses predictive value as more and more neurons reconnect to the original target.

The Endpoint of Regeneration in c-jun−/− Mice

The axonal response in c-jun−/− mice was greatly delayed but not absent. c-jun−/− mice after 15 weeks showed similar motor performance as c-jun+/+ mice after 5 weeks, and this is reflected by a similar extent of retrogradely labeled neurons at these same time points (Figure 4). In wild-type mice, axonal regeneration appears to approach completion 15 weeks after injury, as we have not observed a further increase in motor performance in wild-type mice beyond this time point (our unpublished data). Moreover, 68% of wild-type motoneurons survive the axotomy (Figure 5B), and we observed that 62% of motoneurons are retrogradely labeled 103 days after injury (Figure 4B), thus more than 90% of the surviving neurons have reconnected to their target at this point.

It is unclear whether c-jun−/− mutant motoneurons will eventually accomplish the endpoint of regeneration (defined as the maximal target reinnervation in control animals) or whether reinnervation will remain at a lower level compared to controls. In any case, although c-Jun function is important for the axonal response, there must be alternative molecular pathways that can promote neuronal regeneration in the absence of c-jun, albeit at a much lower efficiency.

C-Jun Is Required for Both Axonal Regeneration and Neuronal Cell Death

Our study strongly supports the perception of c-Jun action as a “double-edged sword” promoting posttraumatic neuronal cell death as well as axonal regeneration (Herdegen et al., 1997). Absence of c-jun in axotomized facial motoneurons interfered with their reconnection to the whisker pad, but c-jun−/− deficiency also prevented the neuronal cell death following facial axotomy. The atrophy of facial motoneurons in c-jun−/− mice may be caused by a combination of these two c-Jun functions. Facial motoneurons that fail to reach their peripheral target would normally die due to lack of trophic support. Without c-Jun, motoneurons that fail to reestablish a functional synapse may survive despite the lack of trophic support, which could explain the atrophy observed in axotomized motoneurons (Figure 5F).

The Molecular Mechanism of c-Jun-Mediated Axonal Growth

Upregulation of growth-associated molecules is considered to play an essential role in transforming normal adult neurons into a regenerating phenotype that supports axonal elongation and/or central sprouting, both types of responses observed in axotomized facial motoneurons (Galiano et al., 2001). Previous studies show that genetic deletion of galanin, α7β1 integrin, and CD44 strongly reduces the rate of axonal regeneration in vivo (Werner et al., 2000; Wynick et al., 2001; L.L. Jones and G.R., unpublished data). The almost complete failure to upregulate these molecules in the axotomized facial motoneurons in the absence of neuronal c-Jun strongly suggests their involvement in mediating c-Jun-dependent axonal outgrowth. Importantly, the promoters of galanin and CD44 contain identified AP-1 sites, which could mediate c-Jun induction (Anouar et al., 1999; Lee et al., 1993). However, it is likely that there are additional, as yet unidentified, transcriptional targets of c-Jun that are necessary for some aspects of the axonal response. Transcriptional profiling of c-jun+/+ and c-jun−/− mice before and after axotomy may therefore result in the identification of novel molecules involved in neuronal regeneration.

The fact that c-Jun was required to upregulate growth-associated molecules such as CD44 in five different axotomy models (Figures 7A–7D and Supplemental Figure S3 at http://www.neuron.org/cgi/content/full/43/1/57/DC1) underscores the overall importance of this transcription factor in turning injured neurons into a regenerating phenotype. As c-Jun appears to be a key regulator of the axonal reaction, pharmacological augmentation of c-Jun activity could represent a promising therapeutic strategy to facilitate axonal regeneration in patients with nerve trauma.

Experimental Procedures

Animals

Mice carrying a floxed c-jun allele, c-junfl (Behrens et al., 2002), were crossed with animals expressing cre recombinase under the control of nestin promoter (Tronche et al., 1999) and then crossed again to generate the c-junfl:nestin-cre−/− mutant mice, in which both c-jun alleles are inactivated in cells derived from embryonic neuroepithelium (c-jun−/−). Compound c-junfl−/− mice were in a mixed 129Ola/C57BL6/FVBN genetic background. Sibling animals lacking the cre transgene, with functional, unrecombined homozygous c-junf−/− (c-jun−/−), served as controls.

Surgery

The right facial nerve fibers (including the retroauricular branch) of 2–3-month-old mice were cut at the stylomastoid foramen under tri-brom-ethanol (Avertin) anesthesia. All animals were fixed by formaldehyde perfusion under deep ether anesthesia (Moller et al., 1996). Whisker pad reinnervation was assessed by applying 15 µl of 4% Fluorogold (Fluorochrome, Denver, CO) into both whisker pads 28 and 103 days after unilateral nerve cut and counting the number of fluorescently labeled motoneurons in the injured and contralateral facial motor nuclei 72 hr after Fluorogold application (Werner et al., 2000).
Immunohistochemistry

Tissue sections (20 μm) were stained overnight with primary antibodies against α7, αM, CGRP, c-Jun (Santa Cruz), galanin, GFAP, MAP2, or NeuN, as described in previous studies (Galiano et al., 2001; Werner et al., 2000), and visualized using biotinylated goat-antirabbit or anti-rat immunoglobulins, ABC peroxidase complex (Vector), and staining with diaminobenzidine and hydrogen peroxide. A Sony 3 CCD video camera was used to obtain 8 bit digital images (based on a 0–255 scale of optical luminosity values) of the antibody stained sections. The immunohistochemical staining was quantified using the OPTIMAS 6.2 imaging system (Media Cybernetics Inc, Silver Spring, MD) and the Mean-SD algorithm. The overall mean optical luminosity value for the antibody staining intensity (SI) was determined for each facial motor nucleus, and subsequently the SI of the adjacent glass was subtracted.

Southern and Western Blot Analysis

For Southern blots, 10 μg of genomic DNA was digested with the indicated restriction enzymes, and a 0.6 kb BamHI fragment from the c-Jun promoter region was used as a probe (Behrens et al., 2002). Western blot analysis was performed according to standard procedures as described (Nateri et al., 2004) using antibodies specific for c-Jun (Santa Cruz) and β-actin (loading control, Sigma).

Neuronal Cell Counts

The brain tissue was postfixed for 2 days in 4% buffered formaldehyde, cut in a cryostat at -15°C, and the 25 μm frontal tissue sections collected throughout both facial nuclei (ax and co) and stained with Toluidine blue (Nissl). Neuronal cell counts were corrected for neuronal cell body size using Abercrombie correction.

Watermaze and Open Field

The testing was done in a circular pool made of white polypropylene (diameter, 150 cm; wall height, 50 cm), filled to a height of 16 cm with water made opaque by the addition of milk and maintained at 24°C–26°C. A 16 × 16 cm target platform of white wire mesh was placed 0.5 cm below the water surface in the NW, NE, SE, or SW quadrant at 35 cm from the wall. Each mouse was trained for a constant platform position during 3 days with six trials per day separated by 30–60 min intervals and lasting maximally 120 s. This acquisition phase was followed by 2 days of reversal training with the platform positioned in the opposite quadrant. The first 60 s of the first reversal trial served as probe trial to test spatial retention. The pool was illuminated by indirect diffuse room light (four 40 W bulbs, 12 lux).

The home cage rack was brought to the test room 30 min before the experiment, and the apparatus was thoroughly cleaned with 70% ethanol before releasing each animal. The round openfield arena had a diameter of 150 cm, a smooth floor of white plastic, and 35 cm high sidewalls made of white polypropylene. It was illuminated by indirect diffuse room light (four 40 W bulbs, 12 lux). Each subject was released near the sidewalk and was observed for 10 min. The same procedure was repeated the following day, resulting in a total observation time of 20 min.

Animals were video tracked using a Noldus EthoVision 1.96 system (Noldus Information Technology, Wageningen NL, www.noldus.com) at 4.2 Hz and 256 × 256 pixel spatial resolution. Raw data were transferred to public domain software Wintrack 2.4 (www.dpwolfer.ch/wintrack [Wolfer et al., 2001]) for further analysis.

Acknowledgments

We are grateful to J.J. Haigh and G.P. Schiavo for the critical reading of the manuscript, and we thank U. Mayer for the α7-integrin antibody. This study was supported by the BBMRI grant S2029G (G.R.) and the Nathalie Rose Barr PhD Studenship Award NRBS08 from the International Spinal Research Trust (M.M., G.R.). L.R.-S. acknowledges support from an EU Marie-Curie fellowship. The London Research Institute is funded by CR-UK.

Received: September 24, 2003
Revised: April 27, 2004
Accepted: June 4, 2004
Published: July 7, 2004

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