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Caspase-3 is a target gene of c-Jun:ATF2 heterodimers during apoptosis induced by activity deprivation in cerebellar granule neurons

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ABSTRACT

Caspase-3, a key executor of neuronal apoptosis, is up-regulated and activated during apoptosis induced by activity deprivation in cerebellar granule neurons (CGNs). However, the transcriptional mechanism regulating caspase-3 during CGN apoptosis remains unknown. Here, we show that the *caspase-3* gene is transactivated and its induction is preceded by c-Jun NH_2-terminal kinase (JNK)/c-Jun:ATF2 pathway activation following activity deprivation in CGNs. We observed that *caspase-3* induction is abolished by pharmacological inhibition of the JNK/c-Jun:ATF2 pathway. Destroying c-Jun:ATF2 heterodimers with dominant negative mutants of c-Jun and ATF2 or knockdown by small RNA interference reduced *caspase-3* promoter activity and mRNA level. Furthermore, chromatin immunoprecipitation showed increased binding of c-Jun:ATF2 heterodimers to the *caspase-3* promoter in response to activity deprivation *in vivo*. Site-directed mutagenesis of the *caspase-3* promoter revealed that *caspase-3* transcriptional activation depends primarily on an ATF site −233 to −225 nucleotides upstream of the start site. Taken together, these data demonstrate that *caspase-3* is a target gene of c-Jun:ATF2 heterodimers during apoptosis induced by activity deprivation in CGNs.

Caspases are a family of cysteinyl aspartate-specific proteases that are highly conserved in multicellular organisms and function as downstream effectors of apoptosis [16]. Caspase-3 is an important member of this family and exerts critical functions in a variety of neuronal death paradigms including activity-deprived CGNs [1,2], nerve growth factor (NGF)-deficient sympathetic neurons [23], cortical neurons with DNA damage [8], and dopaminergic neurons in Parkinson's disease [4]. During apoptosis, caspase-3 is activated by sequential processing from its p32 pro-enzyme form to a small p12 and a large p17 subunit, which constitute the activated form of the enzyme, a major executor of apoptosis [16].

In addition to proteolytic activation regulation, the level of *caspase-3* mRNA is related to the fate of the cell and disease progression [12]. Previous studies have demonstrated a significant increase in the transcription of *caspase-3* after activity deprivation in CGNs [2,3] and suggest that its expression may be induced by a decrease in calcium and cyclic AMP [14]. Analysis of the *caspase-3* promoter has revealed several Sp1 binding sites [10]; Ets-1-like elements [10]; NFκB, AP1, and p53 binding sites [17]; HIF-1 binding sites [20]; PHF10 binding sites [22]; and putative progesterone response elements [7] that can modulate *caspase-3* transcriptional activity in a cell type-dependent manner. However, the transcriptional mechanism regulating *caspase-3* during CGN apoptosis remains largely unknown.

We have previously reported that activity deprivation leads to JNK-dependent transactivation of ATF2 and c-Jun and that c-Jun:ATF2 heterodimers activate the transcription of their target genes to promote neuronal apoptosis [26]. Although the JNK/c-Jun:ATF2 signaling pathway and caspase-3 have been implicated in CGN apoptosis induced by activity deprivation [6], there is no evidence for a functional relationship between *caspase-3* transcription and the JNK/c-Jun:ATF2 signaling pathway. In this study, we demonstrate that activity deprivation leads to JNK/c-Jun:ATF2-dependent transactivation of *caspase-3*, a target gene of c-Jun/ATF2 heterodimers during activity deprivation-induced apoptosis in CGNs.

Rat CGNs were prepared from 7- to 8-day-old Sprague–Dawley rat pups (15–19 g) as described previously [11,26]. After 7 days

Abbreviations: CGN, cerebellar granule neuron; ChIP, chromatin immunoprecipitation; DIV, days *in vitro*; DMSO, dimethylsulfoxide; JNK, c-Jun NH_2-terminal kinase; 25K, 25 mM KCl; 5K, 5 mM KCl; MOI, multiplicity of infection; Q-PCR, quantitative PCR.

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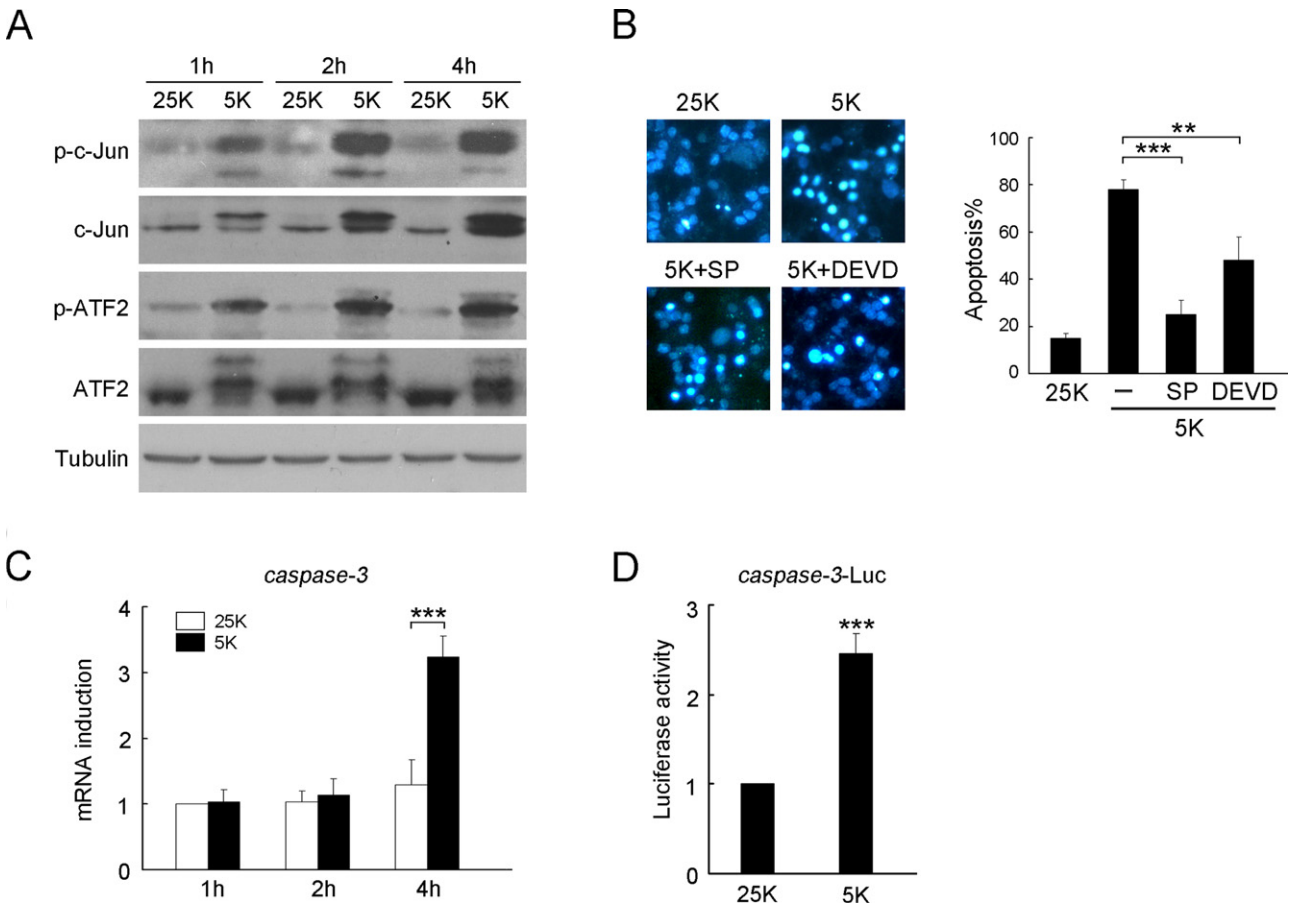

Fig. 1. The JNK/c-Jun:ATF2 pathway is activated and *caspase-3* is up-regulated during activity-deprivation induced apoptosis in CGNs. (A) DIV7 CGNs were maintained in medium containing 25K or 5K for 1 h, 2 h or 4 h and then subjected to Western blotting using the indicated antibodies. (B) Representative images of CGNs incubated in media containing 25K, 5K, 5K plus 10 μM SP600125 (SP), or 50 μM Z-DEVD-fmk (DEVD) for 8 h are shown (left panel). Neurons were stained with Hoechst 33258 to visualize nuclei. Apoptosis was quantified by scoring the percentage of neurons with pyknotic nuclei (right panel). (C) After maintenance in 25K or 5K for the indicated time (1 h, 2 h, 4 h), CGNs were subjected to Q-PCR using *caspase-3*- and *β-actin*-specific primers. (D) DIV6 neurons were co-transfected with 1 μg of *caspase-3*-Luc together with 200 ng of pCMV-RL. Twenty-four hours after transfection, neurons were switched to media containing 25K or 5K for 10 h. The levels of luciferase activity were determined and normalized to Renilla luciferase activity. Data represent the mean ± SEM of four independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

in vitro (DIV7), neurons maintained in serum-containing media with 25 mM KCl were incubated in serum-free media containing either 25 or 5 mM KCl (25K [control] or 5K [activity deprivation]). For inhibitor experiments, CGNs were maintained in the presence or absence of the inhibitors SP600125 (Calbiochem) or Z-DEVD-fmk (Biomol). Cells that were not treated with inhibitors received dimethylsulfoxide (DMSO) as a control; the final concentration of DMSO was less than 0.1%.

Western blot analysis was performed as previously described [11,26]. The following primary antibodies were used: anti-phospho-JNK, anti-phospho-c-Jun (Ser73), anti-phospho-ATF2 (Thr69/71) and anti-ATF2 from Cell Signaling Technology; anti-c-Jun from BD Biosciences; and anti-tubulin from Sigma–Aldrich.

Q-PCR was performed in triplicate on an iCycler IQ5 PCR machine (Bio-Rad) with iQ5 SYBR Green Supermix (Bio-Rad) according to the manufacturer's instructions. PCR cycling conditions were as follows: initial denaturation at 95 °C for 3 min followed by 40 cycles of 95 °C for 10 s, 30 s of annealing and extension (annealing temperature adapted for the specific primer set used). The following primer pairs were used: *c-jun* (forward: 5′-TGGGCACATCACCACTACAC-3′ and reverse: 5′-AGTTGCTGAGGTTGGCGTA-3′); *caspase-3* (forward: 5′-ACTTCCATAAAAGCACTGGAATGTC-3′, and reverse: 5′-GCCATGAATGTCTCTCTGAGGTT-3′); *β-actin* (forward: 5′-AGCCATGTACGTAGCCATCC-3′; reverse: 5′-GTGGTGGTGAAGCTGTAGC-3′).

The BS/U6 vector was used to express small hairpin RNAs (shRNAs) as described previously [11,26]. Two 19-nucleotide c-jun shRNAs (shc-juna and shc-junb) were designed to target the sequences 5′-ACAGGTGGCACAGCTTAAA-3′ and 5′-AGTCATGAACCACGTTAAC-3′ in the coding sequence region of *c-jun* mRNA. Two 19-nucleotide ATF2 shRNAs (shatf2a and shatf2b) were designed to target the sequences 5′-GAAGAAGTGGGTTTGTTTA-3′ and 5′-GCTATTCCTGCATCAATTA-3′ of *atf2* mRNA, respectively [11,26].

A fragment spanning the area from −1858 to +87 nucleotides relative to the transcription start site of the rat caspase-3 genomic sequence was produced by PCR and fused to the promoterless firefly luciferase gene in the pGL3-Basic vector (Promega) to generate *caspase-3*-Luc. The mutant ATF site (TTTACCTCAAG to AGGACCTCGTC) [18] was introduced into the *caspase-3*-Luc plasmid by overlap extension PCR [11]. CGNs were transfected with *caspase-3*-Luc or pGL3 together with a Renilla luciferase gene under the control of the constitutively active cytomegalovirus (CMV) promoter (CMV-RL) using the calcium phosphate co-precipitation method described previously [11,26]. The CGNs were then lysed, and these lysates were subjected to dual luciferase assay (Promega). The levels of firefly luciferase activity were measured and normalized to Renilla luciferase activity [11,26].

The recombinant adenoviruses Ad-GFP and Ad-FLAG-c-Jun-Δ169 were purified and used as described previously [11]. DIV5 neurons were infected with Ad-GFP or Ad-FLAG-c-Jun-Δ169 at a