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Letter to the Editor

Regulation of Hsp27 expression and cell survival by the POU transcription factor Brn3a

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Dear Editor,

The POU family transcription factor Brn3a is expressed in specific regions of the developing and adult nervous system.[1] Both *in vivo* and *in vitro* studies demonstrate a critical role for Brn3a in the survival and specification of sensory neurons.[2,3] In the sensory neuron-derived cell line ND7 Brn3a is associated with neurite outgrowth, cell cycle arrest, differentiation and protection against apoptosis. cAMP or serum withdrawal (SW) causes the upregulation of Brn3a expression in ND7 cells and correlates with the transactivation of target promoters associated with survival or differentiation.[4,5]

Hsp27 is a member of the small stress protein (sHsp) family and can confer resistance to a number of cytotoxic stresses, including heat, ischemia, anticancer drugs, oxidative stress and excitotoxicity.[6] Hsp27 is highly expressed in neuronal cells and is shown to protect against neuronal cell apoptosis.[7] Mehlen *et al*.[8] reported an increase in Hsp27 expression and Hsp27-dependent protection against apoptosis during dopamine-induced, cAMP-dependent differentiation of rat olfactory neuroblasts. Since there are direct parallels between survival and differentiation in these two experimental systems, we hypothesised that Hsp27 may represent a downstream target of the Brn3a transcription factor during neuronal cell survival and differentiation.

Investigation of the DNA sequences of the mouse and human Hsp27 promoters revealed an A–T-rich sequence TTGCCATTAATAG, which corresponded to a consensus Brn3a binding site[9,10] and consisting of a core ATTAAT element intersecting two half oestrogen response elements (EREs) at −87 to −91 and −71 to −73. We subcloned the Hsp27 promoter region[11] into the pGL2-luciferase reporter plasmid and co-transfected this into ND7 cells along with plasmids expressing full-length Brn3a(l), which contains the N-terminal transactivation and POU DNA binding domains, Brn3a(s) that lacks the N-terminal transactivation domain and the Brn3a POU domain in isolation. Co-transfection with the oestrogen receptor (ER) was also used as a positive control. As shown in Figure 1a, the wild-type promoter was activated 29-fold by full-length Brn3a(l), compared to a seven-fold activation by co-expression of the ER, but not by Brn3a(s) or the POU domain alone. This suggests that the N-terminal activation domain of Brn3a is required. The long (l) and short (s) isoforms of Brn3a result from alternate promoter usage.[12] The 46 kDa Brn3a(l) contains an N-terminal activation domain not present in the 35 kDa Brn3a(s). Interestingly, while some promoters are activated by both Brn3a isoforms (i.e. SNAP25; synaptophysin) others require the N-terminal activation domain, particularly those involved in cell survival, such as Bcl-2 and Bcl-X_L.[13] Site-directed mutagenesis of three nucleotides within the CATTAAT sequence to CGCCAAT within the context of the intact promoter abolished the ability of Brn3a(l) to transactivate the promoter (Figure 1b), but did not affect basal promoter activity. An enhanced activation of the promoter by ER in this case is consistent with the observation that Brn3a is able to interact with ER and represses its effect on an ERE-containing promoter. Furthermore, activation of the promoter by ER in the presence of the mutated site indicates that promoter activity is otherwise intact and that the CATTAAT site is not simply functioning as a TATA box.

We investigated whether this effect of Brn3a was mediated by binding to the CATTAAT sequence within the proximal Hsp27 promoter by performing EMSAs using labelled oligonucleotides corresponding to the putative Brn3a binding site (5′-TTG CCA TTA ATA GAG-3′) and *in vitro*-translated (IVT) Brn3a. Incubation of labelled probe with IVT Brn3a resulted in two major bands that were specifically competed upon addition of unlabelled ('cold') oligonucleotide, but not by a nonspecific oligonucleotide or an oligonucleotide in which the Brn3a site was mutated (Figure 1c). Moreover, Brn3a failed to bind the labelled mutant probe.

Functional analysis of whether Brn3a modified endogenous Hsp27 levels in ND7 cells showed that either SW alone or overexpression of Brn3a(l) clearly induced expression of Hsp27 (Figure 1e and f). This induction was further potentiated by overexpression of Brn3a followed by SW. Brn3a(l) also induced expression of the Brn3a(s) isoform as previously described.

The effect of increased Brn3a and Hsp27 expression on survival and apoptosis following SW in ND7 cells was assessed by staining of the cells with annexin V and propidium iodide (PI) and subsequent FACS analysis. Annexin V staining (Figure 1e and f) showed that SW caused a substantial increase in the number of cells undergoing apoptosis (total annexin V positive cells mean $\pm$ S.E.M: pLTR 12.9 $\pm$ 4.0% *versus* pLTR + SW 65.8 $\pm$ 2.8%, $P \leq 0.01$). Overexpression of Brn3a(l) significantly reduced the number of cells that were undergoing apoptosis, as indicated by a substantial reduction in both annexin V positive subpopulations (3a 21.4 $\pm$ 3.1%, $P \leq 0.01$).

This observation was supported by PI staining and cell cycle analysis. SW in (control) pLTR transfected cells resulted

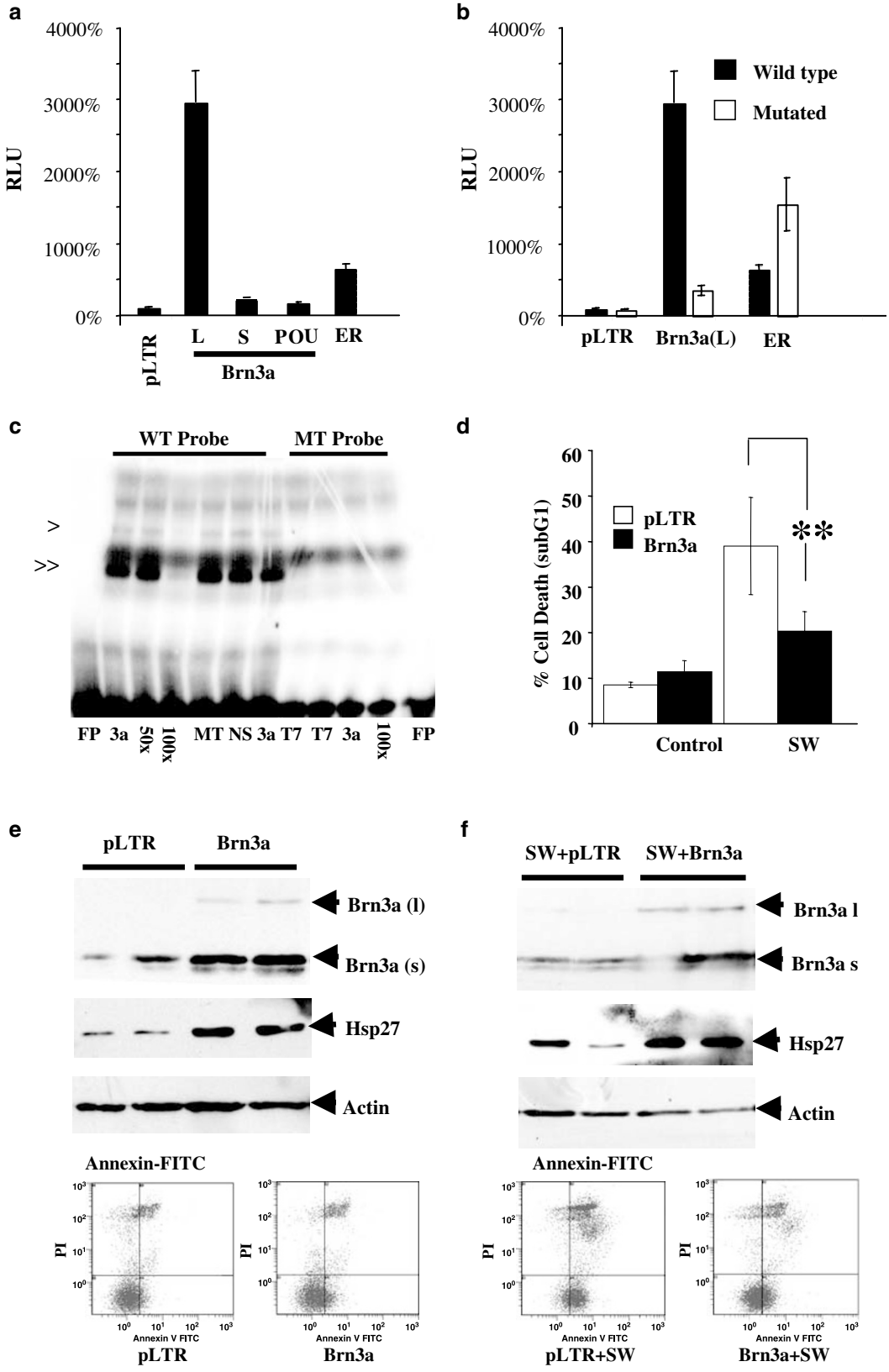
a
4000%
3000%
2000%
1000%
0%
RLU
pLTR
L
S
POU
ER
Brn3a
b
4000%
3000%
2000%
1000%
0%
RLU
Wild type
Mutated
pLTR
Brn3a(L)
ER
c
WT Probe
MT Probe
FP 3a 50x 100x MT NS 3a T7 T7 3a 100x FP
d
60
50
40
30
20
10
0
% Cell Death (subG1)
pLTR
Brn3a
**
Control
SW
e
pLTR
Brn3a
Brn3a (l)
Brn3a (s)
Hsp27
Actin
Annexin-FITC
PI
Annexin V FITC
pLTR
Brn3a
f
SW+pLTR
SW+Brn3a
Brn3a l
Brn3a s
Hsp27
Actin
Annexin-FITC
PI
Annexin V FITC
pLTR+SW
Brn3a+SW

Figure 1

in a significant loss of cells in G_0/G_1 phase of the cell cycle and an increase in the amount of sub-G_1 DNA indicative of substantial total cell death (necrosis plus apoptosis: Figure 1d) (pLTR + SW 42.8 ± 5.7% *versus* pLTR 13.9 ± 1.2%, $P \leq 0.01$). Transfection with Brn3a(l) resulted in an increase in the survival (3a + SW 22.5 ± 2.9%, $P \leq 0.01$) with the surviving cells showing an increase in the proportion of cells in G_0/G_1 phase of the cell cycle (3a + SW 42.1 ± 2.2% *versus* pLTR + SW 27.8 ± 3.6%, $P \leq 0.05$), which correlates with increased Hsp27 expression (Figure 1e and f). Brn3a alone in the presence of serum resulted in a slight increase in the number of cells in G_0/G_1 phase of the cell cycle (3a 49.9 ± 2.8% *versus* pLTR 47.1 ± 4.3%, P = NS), as previously described.[5] Therefore, Brn3a and subsequent induction of Hsp27 appears to protect ND7 cells against the pro-apoptotic effects of SW and increase the proportion of surviving cells in G_0/G_1 phase of the cell cycle.

In summary, we found that Brn3a strongly activated the Hsp27 promoter by direct binding to a consensus element. Overexpression of Brn3a resulted in increased levels of endogenous Hsp27 expression and this was associated with increased cell survival and percentage of cells arrested in G_0/G_1 phase of the cell cycle. This is interesting as transcriptional and post-tranlational regulation of Hsp27 is necessary for neuronal survival. However, little is known about how the Hsp27 gene is transcriptionally regulated either developmentally or following stress. The ability of Brn3a to strongly induce Hsp27 may represent an important pathway for regulation of Hsp27 expression in Brn3a expressing cell types.

Brn3a appears to regulate the expression of two classes of genes, that is, those encoding proteins necessary for neuronal differentiation including SNAP25, α-internexin, synaptophysin and neurofilament and those encoding proteins necessary for survival/protection against apoptosis such as Bcl-2 and Bcl-X_L.[5] It is likely that Brna3a mediated survival effects are co-dependent on Bcl-2 and Hsp27, which may function co-operatively within the mitochondrial pathway. Hence, manipulation of either Hsp27 or Bcl-2 will block the protective effects of Brn3a, indicating that both are necessary, but neither are sufficient for protection. Thus, Brn3a appears to be an important regulatory factor for Hsp27 in sensory neuronal cells.

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Figure 1 (**a**) Strong activation of the wild-type Hsp27 promoter by Brn3a. The human Hsp27 promoter cloned into the pGL2-luciferase reporter vector was co-transfected into ND7 cells with expression vectors encoding Brn3a(l), Brn3a(s), the Brn3a POU domain, the oestrogen receptor (ER) or empty vector (pLTR). Renilla TK luciferase was used as a control for transfection efficiency. (**b**) Mutation of the Brn3a binding site abolishes activation. Reporter constructs with the wild-type Brn3a binding site (CATTAAT) (filled bars) or with the Brn3a binding site mutated to CGCCAAT (open bars) were co-transfected into ND7 cells with expression vectors encoding Brn3a(l), the oestrogen receptor (ER) or pLTR. For (**a**) and (**b**), luciferase activity is expressed as relative luciferase units (RLU) corrected to the Renilla TK luciferase activity. Data are expressed as % of pLTR (set at 100%) and as mean + S.D. pooled from three independent experiments. (**c**) Brn3a binds to the consensus site. EMSA was performed using IVT Brn3a(l) and a labelled d/s oligonucleotide corresponding to the Brn3a binding site in the human Hsp27 promoter. Lane 1: Free labelled probe (FP); lanes 2–7: IVT Brn3a(l) protein. Lane 2: Brn3a(l) (3a); lane 3: competition with 50 × molar excess cold probe (50 ×); lane 4: competition with 100 × molar excess cold probe (100 ×); lane 5: competition with unlabelled mutant probe (MT); lane 6: competition with unlabelled nonspecific oligonucleotide; lane 7: T7 control translated protein. Lanes 8–11: labelled oligonucleotide in which the Brn3a binding site was mutated. (**d**) Overexpression of Brn3a(l) protects against SW. ND7 cells were transiently transfected with empty vector (pLTR) or vector expressing Brn3a(l) and either maintained in full growth medium (Control) or serum-free medium (SW) for 48 h. After 48 h, cells were labelled with PI to stain DNA, harvested and analysed by FACS. Total cell death was assessed as the amount of sub-G1 DNA. (**e** and **f**) Overexpression of Brn3a(l) induces Hsp27 and protects against apoptosis. ND7 cells were transiently transfected with pLTR or pLTR-Brn3a(l) and either maintained in full growth medium (**e**) or serum-free medium (SW) (**f**) for 48 h. After 48 h, the cells were harvested for SDS-PAGE and Western blotting blots were probed with monoclonal anti-Brn3a antibody, polyclonal anti-actin antibody as a control for total protein loading or polyclonal anti-Hsp27 antibody. Alternatively, 48 h after treatment cells were dual labelled with PI- and FITC-conjugated annexin V to identify apoptotic *versus* necrotic cells and analysed by FACS