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The novel neurotrophin-regulated neuronal development-associated protein, NDAP, mediates apoptosis

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Abstract **We identified a novel gene and named it, "neuronal development-associated protein (NDAP)". We detected NDAP mRNA presence in most tissues including the brain where it was present in the area from the external granular layer to the multiform layer in the cerebral cortex, and in CA1, CA2, CA3 and the dentate gyrus in the hippocampus. Its expression increased transiently in primary cultures of 2–4 day neurons and 1–2 week astrocytes and was significantly reduced in older cultures. Treatment by the neurotrophin, NT-3, significantly attenuated the decline of NDAP in neurons from days 2 to 10, whereas growth factors such as GDNF and insulin, and high potassium levels did not. To elucidate the effects of neurotrophins, we treated day 5 neurons with NT-3, BDNF or NGF for 48 h. NT-3 and BDNF both inhibited downregulation of NDAP mRNA levels but NGF slightly enhanced the already present downregulation; this effect of NGF was significant when examined in day 3 neurons. To investigate the potential function of NDAP, we over-expressed an NDAP-EGFP fusion protein in 4-week-old astrocytes. The newly expressed NDAP gradually aggregated into membrane-bound structures and eventually led to cell death through apoptosis by 24 h. Significant levels of cell death were also observed in NDAP-EGFP transfected HEK293 cells. Thus maintenance of high NDAP levels may cause apoptosis. The different regulations of NDAP expression by neurotrophins indicate that the expression of NDAP might be a checkpoint for apoptosis during neuronal development.**

Keywords: Neuronal development; Neurotrophins; NT-3; Over-expression; Cell death

1. Introduction

Highly conserved and genetically encoded events are responsible for precisely manipulated developmental programs. The switching on and off of certain genes in a predetermined manner early in embryogenesis ensures appropriate neuronal subtype survival and differentiation [1]. Differential gene expression during development, together with the temporal expression and modulation of growth factors, especially neurotrophins, determine a neuronal fate of maturation or apoptosis [2–4].

Our previous studies demonstrated that neurons in primary cultures prepared from E16 mouse brain cortices become functionally mature between days 4 and 7 [5,6]. RAP-PCR, a means to discover differentially regulated genes, was used to identify differentially expressed mRNA transcripts in these cerebral cortical neurons at various ages (2, 4, 7, and 10 days-old) [6,7]. Among them, we identified one mRNA transcript, which decreased in level from days 4 to 7. We named this novel gene and protein, "neuronal development-associated protein (NDAP)". By screening a cDNA library, the full-length sequence of NDAP was identified and submitted to GenBank as Ndap7 gene (GenBank accession number: AJ308886). From genomic BLAST, the NDAP gene was found to be located on mouse chromosome 2 and the protein product was comprised of 578 amino acids. GenBank database searches showed that NDAP shared a conserved domain with *Saccharomyces cerevisiae* Gpi16 protein and *Homo sapiens* PIG-T protein. These two proteins belong to the transamidase family and are responsible for transferring a glycosylphosphatidylinositol (GPI) tail to newly synthesized proteins [8].

We are interested in the potential role of NDAP in nerve cell development. In this study, we report that the expression of NDAP was regulated by neurotrophins. Cells over-expressing NDAP died of apoptosis, implicating NDAP expression as a potential checkpoint for cell apoptosis during early development.

2. Materials and methods

2.1. Primary cultures and growth factor treatments

Primary cultures of cerebral cortical neurons and astrocytes were prepared from ICR mice as described by Li et al. [6] and Chen et al. [9], respectively. All growth factors except potassium chloride were purchased from Invitrogen (USA). Potassium chloride was purchased from Sigma (USA).

2.2. RAP-PCR, cloning and cDNA library screening

mRNA was isolated from neurons on different days (2, 4, 7, and 10) using the QuickPrep® Micro mRNA Purification System (Pharmacia Biotech, Sweden). RAP-PCR was performed as described by Li et al. [6]. Screening of newborn mouse brain cDNA library in λ ZAP II (Stratagene, USA) was performed according to the manufacturer's instructions. Primers designed to amplify the ORF of full-length NDAP were used to isolate the full-length cDNA from the cDNA pool.

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2.3. NDAP in situ hybridization

Adult mouse brain was fixed in 4% paraformaldehyde at 4 °C overnight. Cryosections of 16 μm were prepared for in situ hybridization. The sections were immersed in hybridization buffer [50% deionized formamide, 1× Denhardt's solution, 4× SSC, 10 mM DTT, 1 mg/ml yeast tRNA, 100 ng/ml digoxigenin (DIG)-labelled antisense cRNA probe (labeled using a DIG labeling kit from Roche, Germany)] at 55 °C for 12 h. After the sections had been washed in 4× SSC and 0.1× SSC, they were incubated in a primary antibody solution (diln 1:2000) containing anti-DIG antibody-alkaline phosphatase conjugate at room temperature overnight. Color development was carried out using Chrimagen Solution (Roche, Germany), followed by serial dehydration with 50–100% alcohol. The sections were permanently mounted in neutral balsam, observed under an optical microscope and stored at room temperature.

2.4. RT-PCR

Total RNA was prepared by TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RT-PCR was carried out according to Li et al. [6]. The sequences of primers were: GAPDH-forward, 5′-TGATGACATCAAGAAGGTGGTGAAG-3′; GAPDH-reverse, 5′-TCCTTGGAGGCCATGTAGGCCAT-3′; NDAP-forward, 5′-AGAGCTCTGGGTCTGGTTCCAAGA-3′; NDAP-reverse, 5′-GCAGGGAGTAGTCGTCAGTGTC-3′. PCR conditions were: 94 °C for 3 min, followed by 29 cycles of 94 °C for 45 s, 59 °C for 45 s, and 72 °C for 1 min; and a final extension at 72 °C for 10 min. PCR products were separated on 2% agarose gels and band intensity was quantified by TotalLab2.0 (Nonlinear, UK).

2.5. Transient transfection of astrocytes and HEK293 cells

pLNCX2-NDAP-EGFP and pLNCX2-EGFP expression vectors were created from pLNCX2 and pEGFP-N1 (Clontech, USA). The full-length coding sequence of NDAP was inserted upstream of EGFP. Transient transfections were performed with Lipofectamine™2000 (Invitrogen, USA) [9]. Nuclei of astrocytes were stained with Hoechst 33342 (2 μg/ml).

Over-expression of NDAP-EGFP caused cell shrinkage and detachment. To assay cell death in detached HEK 293 cells 48 h after transfection, cells in the medium were collected by centrifugation at 1000 rpm for 5 min. The cells were re-plated in poly-D-lysine coated dishes for 12 h. To distinguish dead cells from living cells, the re-plated cells were stained without fixation with propidium idodide (PI) instead of Hoechst 33342. Dead cells exhibit red fluorescence. Cell death rate was calculated as the percentage of dead cells (red) among the transfected (green).

2.6. Statistical analysis

Experiments were repeated a minimum of three times. Statistical analyses were performed using one-way ANOVA with the post hoc Newman–Keuls test for multiple comparisons and two-way ANOVA with the Bonferroni post tests using PRISM®4.0 (GraphPad Software, Inc., USA).

3. Results and discussion

Primary cultures of mouse cerebral cortical neurons of 2, 4, 7, and 10 days were adopted for differential gene expression studies. A differentially expressed cDNA transcript was discovered by RAP-PCR and three clones with overlapping sequences were subsequently isolated from a newborn mouse brain cDNA library. Their assembled sequence was 3.5 kb and contained an open reading frame of 1.737 kb. This was then directly cloned from the cDNA pool and named, "neuronal development-associated protein (NDAP)".

In situ hybridization showed that NDAP mRNA transcripts in the adult mouse brain had specific distributions in the cerebral cortex and hippocampus. NDAP mRNA expression was more evident in the area from the external granular layer to the multiform layer in the cortex (Fig. 1Aa, arrows indicate the lower boundary of external granular and ganglionic layers) and in CA1, CA2, CA3 and the dentate gyrus (DG) in the hippocampus (Fig. 1Ba). No signal was observed in layer I

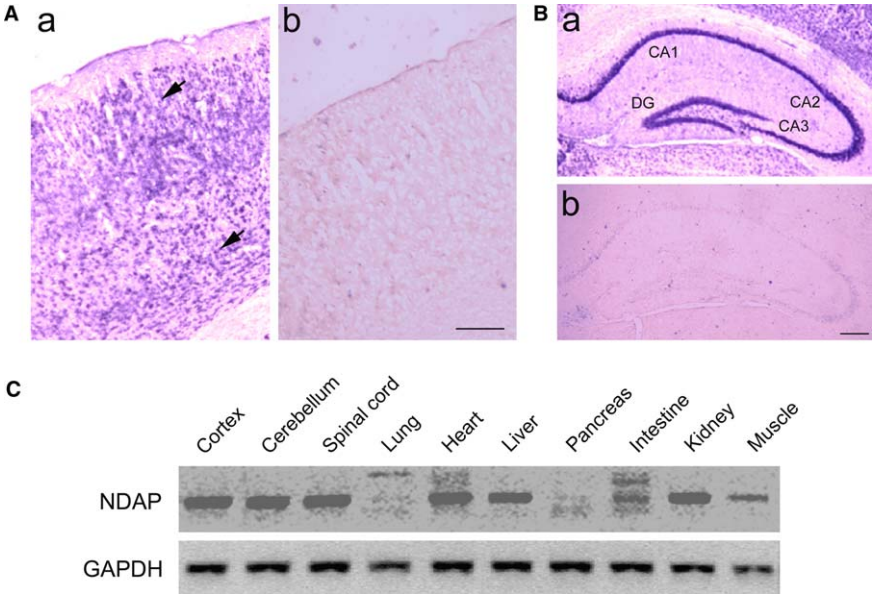

Fig. 1. (A) Localization of NDAP mRNA in mouse cerebral cortex by in situ hybridization. (a) NDAP mRNA transcripts were detected by antisense cRNA probe. High expression of NDAP mRNA was observed in the neurons of layer II (external granular layer) to layer VI (multiform layer). Arrows indicate the lower boundary of layer II (external granular layer) and layer V (ganglionic layer). No positive signal was detected in the layer I (molecular layer). (b) No positive signal could be detected by the sense probe. Bar = 200 μm. (B) In situ hybridization in the hippocampus. (a) High expression of NDAP mRNA transcripts was observed in the neurons in Ammon's horn (CA1, CA2 and CA3) and the dentate gyrus (DG). (b) No signal could be detected by the sense probe. Bar = 200 μm. (C) mRNA expression of NDAP in various tissues. NDAP was detected by RT-PCR in the cortex, cerebellum, spinal cord, heart, liver, intestine, kidney and muscle. Trace levels of NDAP were found in lung and pancreas. GAPDH served as internal control.

(molecular layer) of the cortex (Fig. 1Aa), possibly due to the lack of cell bodies in this layer. Sense probes served as negative controls (Fig. 1Ab and Bb). RT-PCR showed NDAP presence additionally in the spinal cord, heart, liver, intestine, kidney and muscle with trace levels in the lung and pancreas (Fig. 1C). The wide distribution of NDAP mRNA in the brain and other tissues suggested a general function.

In culture, the mRNA expression level of NDAP transiently peaked in day 4 neurons, and then significantly declined on day 7 (~90% of day 4 levels; $P < 0.05$) and day 10 (~87% of day 4 levels; $P < 0.05$) (Fig. 2A), which seemed to follow the functional maturation course of neurons in culture (days 4–7) [5]. Such changes in NDAP mRNA levels were also found in primary cultures of cerebral cortical astrocytes, which peaked at week 2 ($P < 0.05$) and significantly declined afterwards (week 3–4 levels were ~80% of week 2 levels; $P < 0.05$) (Fig. 2B). Developmental expression was also detected in vivo, in embryonic mouse brain by Northern blot. Namely, an increase early in development from E14 to 18, followed by a later decline from E18 to P9 (unpublished data). The high levels of NDAP in younger, and lower levels in older, neurons and astrocytes indicated that a fall in NDAP expression might allow neural cells to enter further phases of differentiation and maturation.

To investigate a role in neuronal development, we examined NDAP gene expression in neuronal cultures under treatment of factors known to affect neuronal development: NT-3, GDNF, insulin, and high potassium levels. GDNF and insulin are involved in stimulating developing neurons and preserving responsiveness in mature neurons [10,11], while potassium regulates the electrical activity of neurons and the growth and branching processes of axons [12]. NT-3 levels and its effects are precisely temporally regulated during development, and various functions have been proposed for its presence very early in neurogenesis [13]. NDAP expression under treatments of GDNF, insulin, or high potassium concentration did not show any difference from that of the untreated control (Fig. 3). However, NT-3 treated cultures did not exhibit the decline in NDAP levels typically seen in neurons from days 4 to 7 (Fig. 3); NDAP mRNA levels remained high in these cultures throughout the observation period (10 days). This reduction in NDAP gene downregulation after NT-3 treatment suggests regulation of NDAP expression by NT-3.

Neurotrophins exert two major effects: promoting neuronal survival and inducing apoptosis, through regulation of the expression of downstream genes [2,3]. To elucidate the role of neurotrophins in regulating NDAP expression, we treated day 5 neurons which have decreasing NDAP levels, with NT-3, BDNF or NGF for 48 h, measured the NDAP mRNA levels, and compared them with those of the untreated control (at day 7). NT-3 and BDNF both precluded the decline of NDAP in day 7 neurons, but only the NT-3 effect was statistically significant (Fig. 4A). Thus NT-3, and possibly BDNF, might exert an upregulatory effect or prevent downregulation of NDAP expression during early neuron development. In contrast, NGF appeared to slightly enhance the decline of NDAP levels in neurons from days 5 to 7. To confirm this downregulatory effect, day 3 neurons were treated with NGF for 24 h. Day 3 neurons in cultures under normal developmental conditions exhibit increasing expression of NDAP (Fig. 2A). The ability of NGF to downregulate gene expression of NDAP in these neurons was statistically significant, thus confirming its downregulatory effect (Fig. 4B).

NT-3, BDNF and NGF are three distinct members of the developmentally timed neurotrophins, whose expression coincides with the onset of neurogenesis and axon elongation [14,15]. During development, they all begin to be expressed simultaneously but the dynamics of their expression vary greatly, and their sequential appearance is critical for neuronal survival and maturation [13,16,17]. NT-3 is the major neurotrophin expressed at high levels in the early embryonic period, and especially in more immature regions. Its mRNA levels decrease while those of BDNF, initially very low, gradually increase. NGF displays comparatively constant mRNA levels, which increase as early axons reach their targets. Previous findings have suggested that neuronal survival responses to this

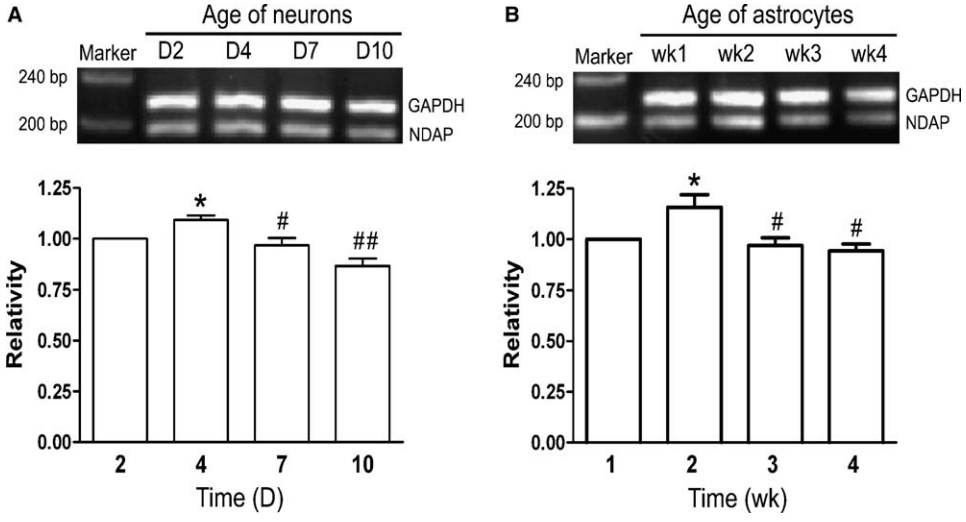

Fig. 2. NDAP mRNA expression profiles. (A) NDAP mRNA expression in primary cultures of neurons of different ages. The level of NDAP increased significantly in the first 4 days and then declined significantly afterwards. $n = 3$. (B) NDAP levels in primary cultures of astrocytes of different ages. The NDAP level peaked at week 2 and then declined significantly afterwards. $n = 3$. D, day; wk, week; *, $P < 0.05$ as compared to control; #, $P < 0.05$ as compared to day 4 neurons or week 2 astrocytes; n, number of independent experiments.

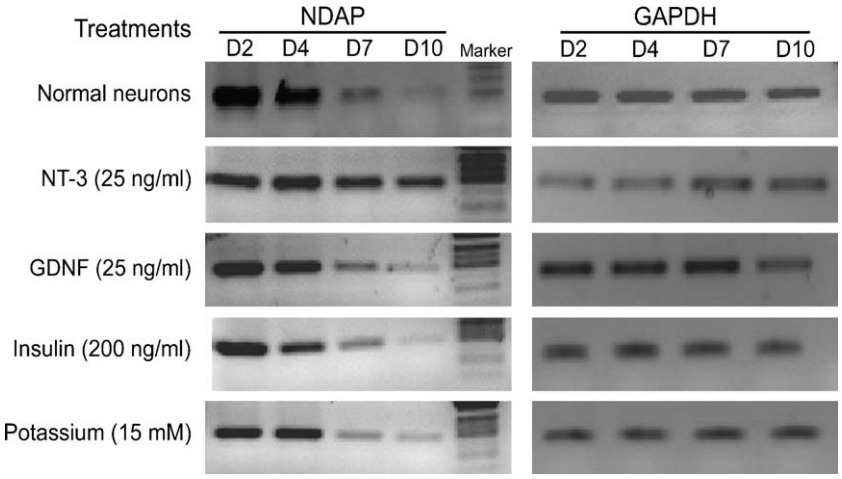

Fig. 3. NDAP levels in neurons grown with NT-3, GDNF, insulin or high potassium. NDAP mRNA levels were measured on days 2, 4, 7, and 10. Only NT-3 prevented the decline of NDAP from day 4 to day 10. D, day.

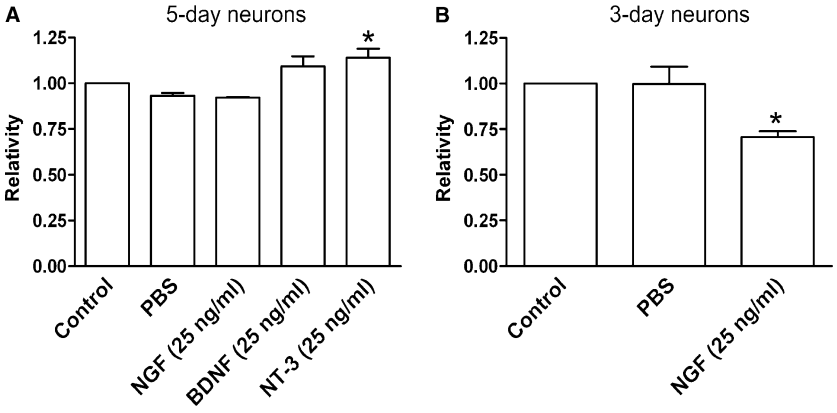

Fig. 4. A: Neurons in day 5 cultures were treated with NGF, BDNF and NT-3 for 48 h. NDAP mRNA levels were measured on day 7. Both NT-3 and BDNF treated neurons had higher NDAP levels, but only that of NT-3 was statistically significant. NGF treated neurons had apparently lower NDAP levels compared with the controls, but this was not significant. $n = 3$. (B) Neurons in day 3 cultures were treated with NGF for 24 h and the NDAP level was measured on day 4. A significantly lower level of NDAP was detected as compared to the untreated control. $n = 3$.

factor may be limited until this stage of neuronal maturation [13,18,19]. The report by Paul and Davies [18] also indicated that the survival response of cultured neurons to specific neurotrophins is highly dependant on the embryonic stage at which the cells are taken. Although in vitro systems might not completely mimic the in vivo situation, our cultured neurons prepared from E16 mice cerebra are immature on day 1 and begin to differentiate, extending neurites to be functionally mature from days 4 to 7. NGF applied on day 3 was able to cause premature downregulation of NDAP indicating that the cultured neurons were NGF-responsive by this stage. Thus BDNF and NT-3 might normally act to maintain NDAP levels before this stage has been reached.

To investigate how high levels of NDAP might affect cells, we over-expressed NDAP in astrocytes and observed their changes under confocal and fluorescent microscopes. Astrocytes in primary cultures were used for their higher transfection efficiency and greater tolerance towards the transfection process than neurons [20]. EGFP transfected astrocytes showed intact and normal morphology (Fig. 5A); whereas NDAP-EGFP transfected astrocytes displayed a shrunken cell body with fewer processes (Fig. 5A). Newly expressed fluorescent NDAP-EGFP was confined to the cytoplasm; but gradually aggregated into membranous structures, possibly those associated with the endoplasmic reticulum (unpublished data). Nuclear condensation was observed in these NDAP-EGFP transfected astrocytes (Fig. 5B). These shrunken astrocytes with condensed nuclei eventually detached from the culture dish and could be positively immunostained for cleaved caspase-3 indicating apoptosis (unpublished data). Similar transfection experiments were performed in the HEK293 cell line. Significantly shrunken cell bodies indicating prominent apoptosis (Fig. 5C, arrowheads) were detected in detached cells collected from HEK293 cell line culture 48 h after transfection with NDAP-EGFP. A death rate of over 40% was found for NDAP-EGFP transfected HEK293 cells compared with 10% for EGFP transfected cells (Fig. 5C).

During development, about half the neurons originally present will undergo apoptosis with mechanisms remaining obscure [1]. Neurotrophins are known to be involved in the regulation of survival during development. The classical neurotrophin hypothesis proposed that the survival of neurons was determined by their access to survival factors [21]. However, this was modified after neurotrophins, including NT-3,

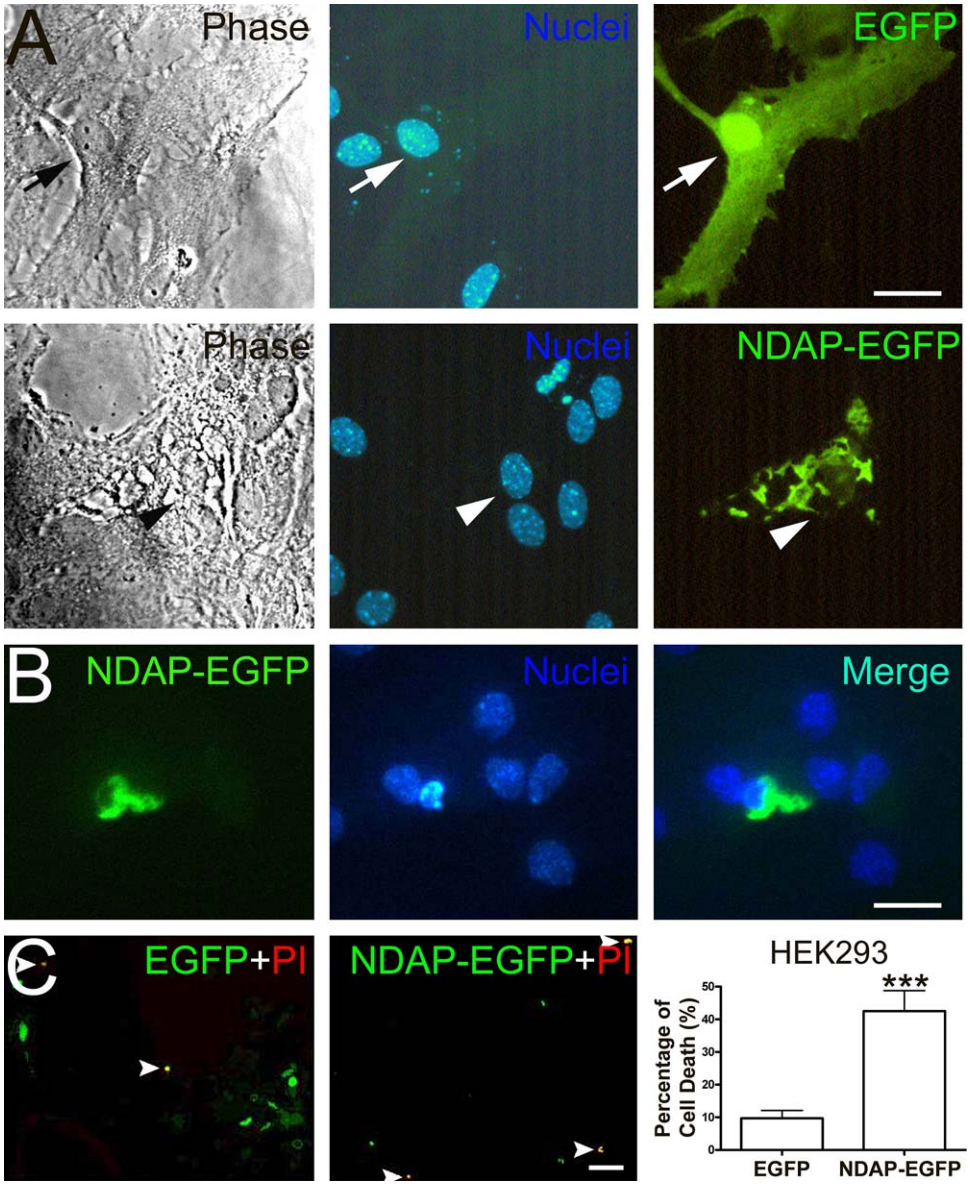

Fig. 5. (A) Astrocytes in 4-week-old cultures were transfected with NDAP-EGFP fusion protein. The transfected cultures were viewed under phase contrast and fluorescent microscopes after 24 h. EGFP transfected astrocytes (arrows) showed intact and normal astrocyte morphology with fluorescence evenly distributed in the cytoplasm (upper panels). NDAP-EGFP transfected astrocytes (arrowheads) showed a shrunken cell body with less cell processes. NDAP was only detected in the cytoplasm, not in the nucleus, and appeared as green fluorescence aggregated in membranous structures. (B) Condensed nucleus located in an NDAP over-expressing astrocyte. (C) NDAP-EGFP transfected HEK293 cells. HEK293 cells were transfected with EGFP and NDAP-EGFP, respectively. Because detachment occurred with NDAP-EGFP transfected cultures, cells in the medium were collected by centrifugation and re-plated in poly-D-lysine coated dishes. PI staining was performed without fixation to identify dead cells. Significantly shrunken cell bodies and higher cell death rate (~40%) were detected in NDAP-EGFP transfected HEK293 cells. Arrowheads indicated PI positive transfected cells. $n = 5$. Bar = 20 μm.

BDNF and NGF, were shown to be capable of inducing apoptosis directly [2,3]. It is now thought that neuronal survival is dependent on the ratio of survival to apoptosis signals from different neurotrophins.

During normal neuronal development, NDAP expression naturally decreases, and our artificial maintenance of high NDAP levels through over-expression led to apoptosis. As a downstream effecter of neurotrophins, NDAP might be involved in neurotrophin regulated apoptosis during development. The initial rise in NDAP mRNA levels in the mouse embryonic brain at days E14-18 (unpublished data), coincides with the time when massive apoptosis (50–70%) is seen in the embryonic cerebral cortex in vivo [22]. This correlation may be significant in the light of our findings.

The facts that NT-3 and BDNF both appeared to upregulate or preclude the decline of NDAP expression and that NGF could enhance this decline, are consistent with a hypothesis that high NDAP expression in more mature developing neurons should be downregulated by NGF to allow entry into further stages of development, such as differentiation and maturation. Neurons maintaining a high level of NDAP would not enter maturation but would die through apoptosis. Thus neurotrophins, NT-3 and BDNF, might oppose rather than mediate neuronal survival and the continued high levels of

NDAP in response to NT-3 and BDNF might represent a process by which developing neurons are eliminated from an inappropriate environment. Thus, NDAP, a novel neurotrophin regulated protein, is a new element and potential checkpoint for survival or apoptosis control during development.

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