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Expression of activity-dependent neuroprotective protein in the brain of adult rats

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Summary. Activity-dependent neuroprotective protein (ADNP) is a VIP-regulated gene, which is essential for brain development. A synthetic peptide (NAP) derived from the ADNP sequence is highly neuroprotective, therefore it has been hypothesised that ADNP has a similar role. ADNP contains classical transcription factor motifs and nuclear localisation domains, but it has also been reported to be secreted and to co-localise with microtubules, indicating that ADNP may have multiple functions. We investigated the pattern of ADNP expression by immunohistology in normal rat brain, in order to generate a framework for future studies examining changes in ADNP expression in response to noxious stimuli or in models of disease. We found widespread ADNP-like immunoreactivity in neurons throughout the rat brain, with the highest expression in the cerebellum, and strong expression in the thalamus, mesencephalon, pons and *medulla oblongata*. ADNP-like immunoreactivity was mainly observed in the cytoplasm of neurons, and fibre tracts were often strongly positive as well. In addition, positive neuronal nuclei were occasionally observed. ADNP-like immunoreactivity was lost in degenerating 'dark' neurons, whereas it appeared to locate to the nucleus in some of the morphologically unaltered adjacent cells. Occasional astrocyte and microglial cells were also positive. We suggest that the widespread expression of ADNP may correlate with the wide-ranging protective effects of NAP, and that the cytoplasmic and axonal localisation of ADNP-like immunoreactivity suggests additional, non-transcriptional functions of ADNP.

Key words: Activity-dependent neuroprotective protein, Dissociated hippocampal cultures, Rat brain

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Introduction

Activity-dependent neuroprotective protein (ADNP) is a vasoactive intestinal peptide (VIP)-regulated gene which is essential for brain development (Pinhasov et al., 2003) and which has been hypothesised to be one of the mediators of VIP-induced neuroprotection. ADNP is a novel protein which contains an eight amino-acid sequence, termed NAP that is similar to an active peptide, ADNF-14, which is derived from a known glial factor, ADNF, a mediator of VIP-related neuroprotection (Brenneman and Gozes, 1996; Brenneman et al., 1998). Since ADNP-14 has strong neuroprotective effects, NAP was also tested, and was found to have astounding neuroprotective effects in a large variety of models (Gozes et al., 2000; Beni-Adani et al., 2001; Spong et al., 2001; Leker et al., 2002, Romano et al., 2002, Ashur-Fabian et al., 2003). NAP has, however, never been observed *in vivo*.

In light of these observations, ADNP has been hypothesised to be a neuroprotective molecule and a mediator of VIP-related neuroprotection, similar to ADNF (Gozes et al., 1999, 2000; Divinski et al., 2004, Steingart and Gozes, 2006). In support of this hypothesis, changes in ADNP mRNA levels have been observed in response to stress or injury (Sigalov et al., 2000; Zaltzman et al., 2004). Furthermore, down-regulation of ADNP increases levels of the tumour suppressor p53 (Zamostiano et al., 2001), further evidence that ADNP might influence cell survival.

The mechanisms through which ADNP might carry out such neuroprotective actions are not fully understood. ADNP mRNA has been detected by northern blot in different parts of the brain including hippocampus, cerebellum and cortex, and in a large number of non-neuronal tissues (Bassan et al., 1999; Zamostiano et al., 2001; Pinhasov et al., 2003; Poggi et al., 2003). So far, however, it remains to be determined

whether the ADNP expression in brain tissues is neuronal or limited to the non-neuronal supporting cells. A detailed description of ADNP protein expression is so far only available for cultured astrocytes, where ADNP was localised to the nucleus and to microtubules (Furman et al., 2004), and for brain regions and organs linked to the female sexual cycle, i.e. arcuate nucleus and vagina (Dangoor et al., 2005; Furman et al., 2005). One potential mode of action of ADNP may be through interaction with microtubules, and indeed NAP has been shown to stimulate the assembly and stabilisation of microtubules (Divinski et al., 2004). An alternative mechanism of activity would be as a transcription factor, since the protein sequence of ADNP contains motifs that are consistent with this role (Bassan et al., 1999; Zamostiano et al., 2001) and in fact nuclear ADNP expression has been demonstrated in astrocytes by immunofluorescence using an anti-NAP antibody (Furman et al., 2004). So far, however, little is known about the pattern of ADNP protein expression in brain parenchymal cells and its subcellular location in these cells.

In this paper, we describe a comprehensive study on the ADNP protein expression pattern in normal rat brain. This provides important groundwork for future studies on the effects of certain conditions or stimuli on ADNP expression and it should contribute to understanding the functional implications of potential changes in ADNP expression in response to stimuli.

Materials and methods

Cell culture and siRNA transfection

HeLa cells were routinely cultured in DMEM high glucose (Autogen Bioclear) supplemented with 10% FBS (Perbio, Hyclone), 1% 200 mM L-glutamine (Biochrom AG), 1% non-essential amino acids (BioWhittaker) and 1% sodium pyruvate (Biochrom AG). $2x10^5$ cells were plated into T25 tissue culture flasks, and transfected with siRNA when at ~70% confluency. Each flask was transfected with 500 pmoles of siRNA reagent, using Oligofectamine transfection reagent (Invitrogen) according to the manufacturer's instructions. Cells were transfected with either non-targeting siRNA 1 (Dharmacon), or with one of two custom-designed siRNAs directed against ADNP, ADNP-11 (guide strand 5'-UUC ACU AUG GAC AUU GCG Gaa, complement strand 5'- CCG CAA UGU CCA UAG UGA Att) and ADNP-12 (guide strand 5'- UUU GGG AGC AAU UAG CAU Caa, complement strand 5'- GAU GCU AAU UGC UCC CAA Att), where N=RNA, n=DNA (Hüsken et al., 2003).

Western Blotting

Transfected cells were homogenised in protein extraction buffer (20% glycerol, 20 mM HEPES, 0.42 M NaCl, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 0.25 mM DTT, 0.5 mM PMSF). The homogenates were centrifuged and the supernatant collected. Total protein content was determined (BCA protein assay, Perbio) and 20 μg aliquots of protein were loaded onto 8% SDS-polyacrylamide gels and separated under standard Laemmli electrophoresis conditions. Gels were blotted onto nitrocellulose membranes using standard transfer conditions. The membranes were probed with rabbit anti-ADNP Ig (Chemicon) at a 1:5000 dilution in TBS + 2% non-fat dried milk, for 1h at room temperature (RT). To confirm equal loading, membranes were stripped and reprobed with rabbit anti-ß-actin polyclonal antibody (Sigma, 1:2000), also for 1h at RT. Detection was carried out by chemiluminescence with the ECL-Plus reagent (Amersham, GEHealthcare).

Dissociated hippocampal cultures

Hippocampi were removed from 2-8 day-old male Wistar rats, trypsinised for 30min at 37°C, washed several times in DMEM high glucose (Autogen Bioclear) plus 10% FBS (Perbio) and gently triturated with a fire-polished pipette. The cells were plated onto glass coverslips coated with poly-D-lysine (Sigma) and laminin (Sigma) using standard methods, and incubated at 37°C/5% CO_2 for 4h. The media was then changed, and 24h after plating the media was replaced by Neurobasal-A media (GibCO/Invitrogen), 2% B-27 supplement (GibCO/Invitrogen) and 0.5 mM L-glutamine (Biochrom AG).

Indirect immunofluorescence

Hippocampal cultures were fixed in 4% buffered paraformaldehyde solution 48h after plating. Non-specific binding was blocked for a minimum of 30min in TBS containing 0.1% Triton X-100, 2% BSA and 0.1% sodium azide. Double staining was carried out sequentially, all antibodies being diluted in the above blocking buffer. Between antibody incubations, cultures were washed in 4 changes of TBS with 0.1% Triton X-100. Incubations with primary antibody were for 1h at RT with either rabbit anti-ADNP Ig (Chemicon) at 1:1000, mouse anti-NeuN IgG1 (clone A60, Chemicon) at 1:30 or mouse anti-GFAP IgG1 (clone GA5, Chemicon) at 1:300 dilution. Negative controls omitted primary antibody. Secondary antibodies used were biotinylated donkey anti-rabbit IgG (Amersham, GEHealthcare) at 1:100 dilution for 1h at RT, followed by streptavidin-Texas Red (Amersham, GEHealthcare) at 1:50 dilution for 30 min at RT, or highly cross-absorbed AlexaFluorTM-488 goat anti-mouse IgG (H+L) (Molecular Probes) at 1:300 dilution for 1h at RT, respectively. The staining pattern was analysed using confocal microscopy (Leica DM1RE2 confocal microscope).

Animals and tissue processing

Five male and five female Sprague-Dawley rats, aged 3 months (weight 270-330g) were killed with

carbon dioxide. Brains were extracted manually, fixed in 10% buffered formalin for 48-72h, cut into 2 mm thick cross-sections with the help of a rat brain blocker and routinely embedded into paraffin wax. 3-5 μm sections were prepared and stained with haematoxylin-eosin for histological evaluation and identification of brain areas, or used for immunohistology.

Immunohistology

Immunohistology for ADNP was performed, using rabbit anti-ADNP Ig (Chemicon) and the peroxidase anti-peroxidase (PAP) method, adapting previously published protocols for polyclonal antibodies (Kipar et al., 1998). Briefly, sections were deparaffinized through graded alcohols. Endogenous peroxidase was blocked by incubation in methanol with 0.5% H_2O_2. After blocking of non-specific binding with 50% swine serum in TBS for 10 min at RT, sections were incubated at 4°C for 15-18h with the primary antibody (1:100 dilution in TBS with 20% normal swine serum), washed with TBS and incubated with swine anti-rabbit IgG (DakoCytomation) and rabbit PAP complex (DakoCytomation), followed by visualisation with diaminobenzidintetrahydrochloride (DAB). Negative controls were incubated with normal rabbit serum instead of the primary antibody. Blocking peptides for the anti-ADNP Ig, however, were not available for absorption to further confirm specificity. A PC12 cell pellet (ATCC No. CRL-1721) was used as positive control. Double staining for ADNP and glial fibrillary acidic protein (GFAP) was performed as described elsewhere, with minor adaptations (Miao et al., 2003). For this purpose, non-specific binding sites were blocked with undiluted goat serum for 20 min prior to incubation with the anti-ADNP antibody, followed by incubation with biotinylated goat-anti rabbit IgG (H+L) (1:100 in PBS, Vector Labs) and avidin biotin complex (ABC) (Vector Labs), with DAB as substrate. Sections were then washed in PBS and incubated with mouse anti-GFAP (1:500 in PBS 1% BSA, GA5, Sigma) for 1h, followed by goat-anti mouse IgG (H+L) (1:100 in PBS, Vector Labs), ABC-alkaline phosphatase (Vector Labs) and the New Fuchsin substrate system. Negative controls were incubated with anti-ADNP or anti-GFAP alone or without both primary antibodies.

Evaluation of ADNP staining

ADNP immunostaining was scored according to intensity of fibre staining and number of positive cells employing at least 10 different brain areas (Table 1). Staining intensity was evaluated semi-quantitatively and immunoreactivity was scored as follows: 0: no ADNP positive cells and/or staining reaction, (+): very few (up to 5) ADNP positive cells per 400x field and/or very faint staining reactions, +: few (up to 15) ADNP positive cells per 400x field and/or faint staining reactions, ++: moderate numbers (≥15) of ADNP positive cells per 400x field and/or moderate staining reactions, +++: high numbers (≥ 30) of ADNP positive cells per 400x field and/or strong staining reactions.

Results

Characterisation of the anti-ADNP antibody

For characterisation, the ADNP antibody was used in a Western analysis of total protein from HeLa cells. In the extract from untransfected HeLa cells, the antibody recognised multiple bands. The predicted full length molecular weight for ADNP is 123.6kDa. In HeLa cells which had been transfected with one of two different siRNAs directed against ADNP, all previously reactive protein species in the molecular weight range of ~95kDa and above disappeared almost completely (Fig. 1) confirming that these bands were specific for ADNP, and that the antibody used for this study specifically recognizes ADNP in western blot analysis.

Overview of in vivo distribution of ADNP-like immunoreactivity

ADNP-like immunoreactivity was widely distributed in the brain of all 10 (five male, five female) Sprague-

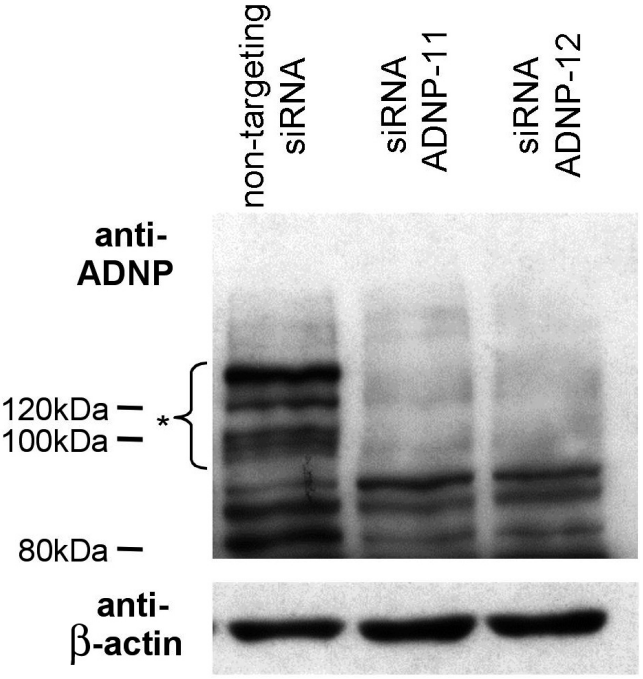

Fig. 1. Western blot for ADNP. HeLa cells were transfected with non-targeting siRNA control, or with two different siRNAs directed against ADNP, ADNP-11 and ADNP-12. The anti-ADNP antibody (AB5878, Chemicon) recognises multiple bands. Several of these bands (*), in the molecular weight range of ~90-150kDa (the expected size for unmodified ADNP being 123.6kDa), are not present in the cells transfected with the siRNAs directed against ADNP, indicating that these bands are specific for ADNP. mRNA analysis confirms a downregulation of ADNP mRNA up to 80% with the siRNAs ADNP-11 and ADNP-12 (data not shown).

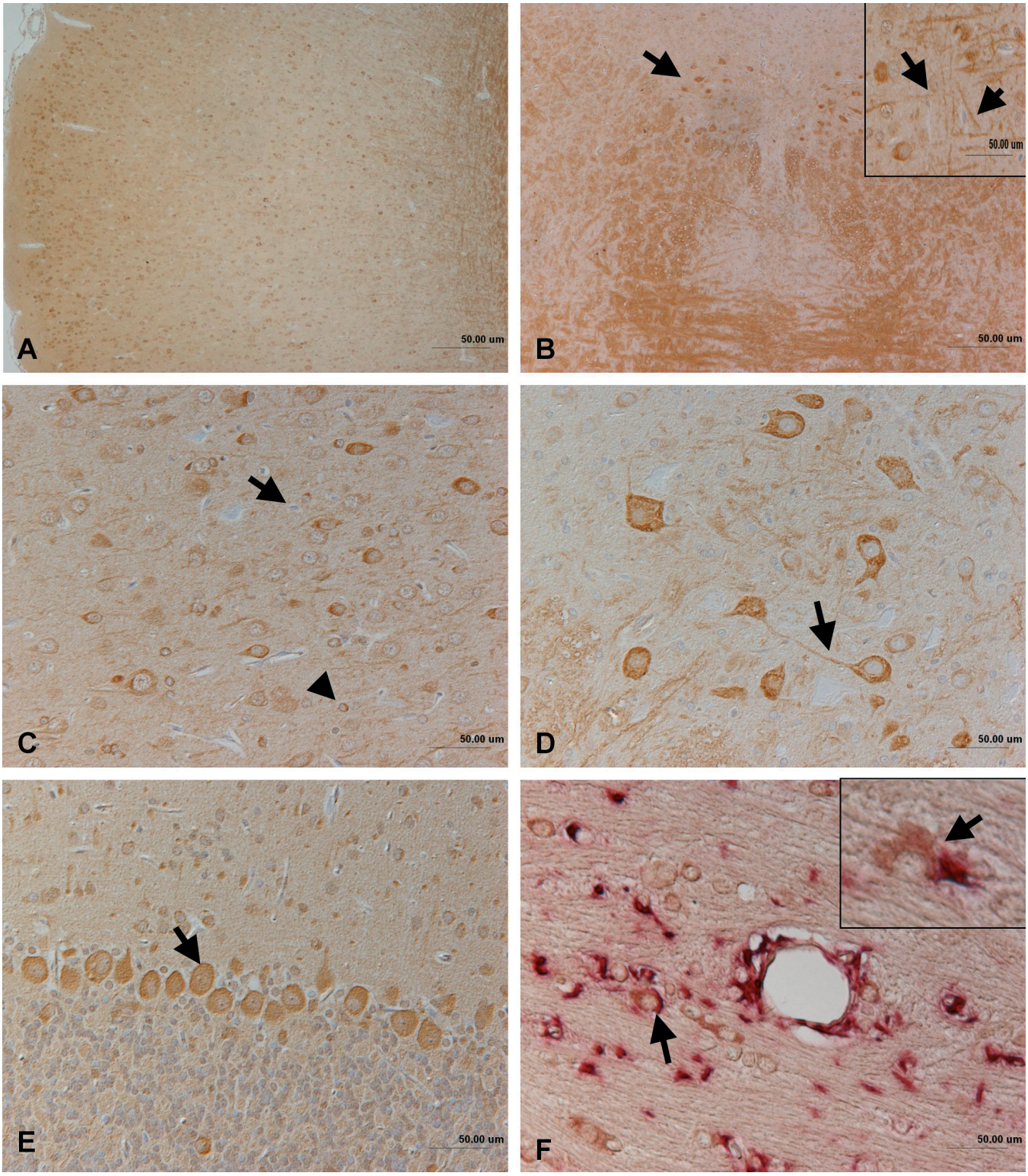

Fig. 2. Expression of ADNP in brains of adult Sprague Dawley rats. **A-F.** Immunohistological demonstration of ADNP-like immunoreactivity, PAP method, Papanicolaou's haematoxylin counterstain. **A.** Widespread expression in the cerebral cortex. **B.** Expression in large neurons (arrow) and fibre tracts of the mesencephalon. Insert: Staining in cell processes (arrows). **C.** ADNP is expressed in the cytoplasm of neurons, smaller cells, most likely glial cells (arrowhead) and satellite cells, but not in the scattered dark neurones (arrow). **D.** ADNP is expressed in the cytoplasm and cell processes (arrow) of larger neurons in the mesencephalon. **E.** In the cerebellum, Purkinje cells exhibit strong cytoplasmic expression (arrow). **F.** Double staining for ADNP (DAB visualisation in brown) and GFAP (New Fuchsin visualisation in red). Note the occasional expression of ADNP and GFAP in astrocytes (arrow). Insert: higher magnification of double-stained astrocyte.

Dawley rats examined without obvious individual or gender differences (Fig. 2A,B). Staining was observed in neurons throughout all structures (Figs. 2C-E, 3A,B).

Neuronal ADNP expression patterns

In neurons, staining was mainly faint to moderate and predominantly cytoplasmic (Fig. 2C,D), often extending into the cell processes (Fig. 2B,D). Occasionally a weak nuclear reaction was observed (Fig. 3A, B). In white matter areas, positive fibre tracts were also seen (Fig. 2B). Neurons of the cerebral cortex or mesencephalon exhibited diffuse immunoreactivity in the perikaryon and also partly in neuronal processes (Fig. 2C,D). Neurons and large motoneurons in the mesencephalon, pons and *medulla oblongata* often showed an intense coarsely granular immunoreaction in the perikaryon (Fig. 2D), whereas Purkinje cells in the cerebellum exhibited a finely granular cytoplasmic reaction (Fig. 2E). Dark neurons, which were occasionally seen in cerebral cortex and hippocampus, were predominantly negative for ADNP (Figs. 2C, 3B).

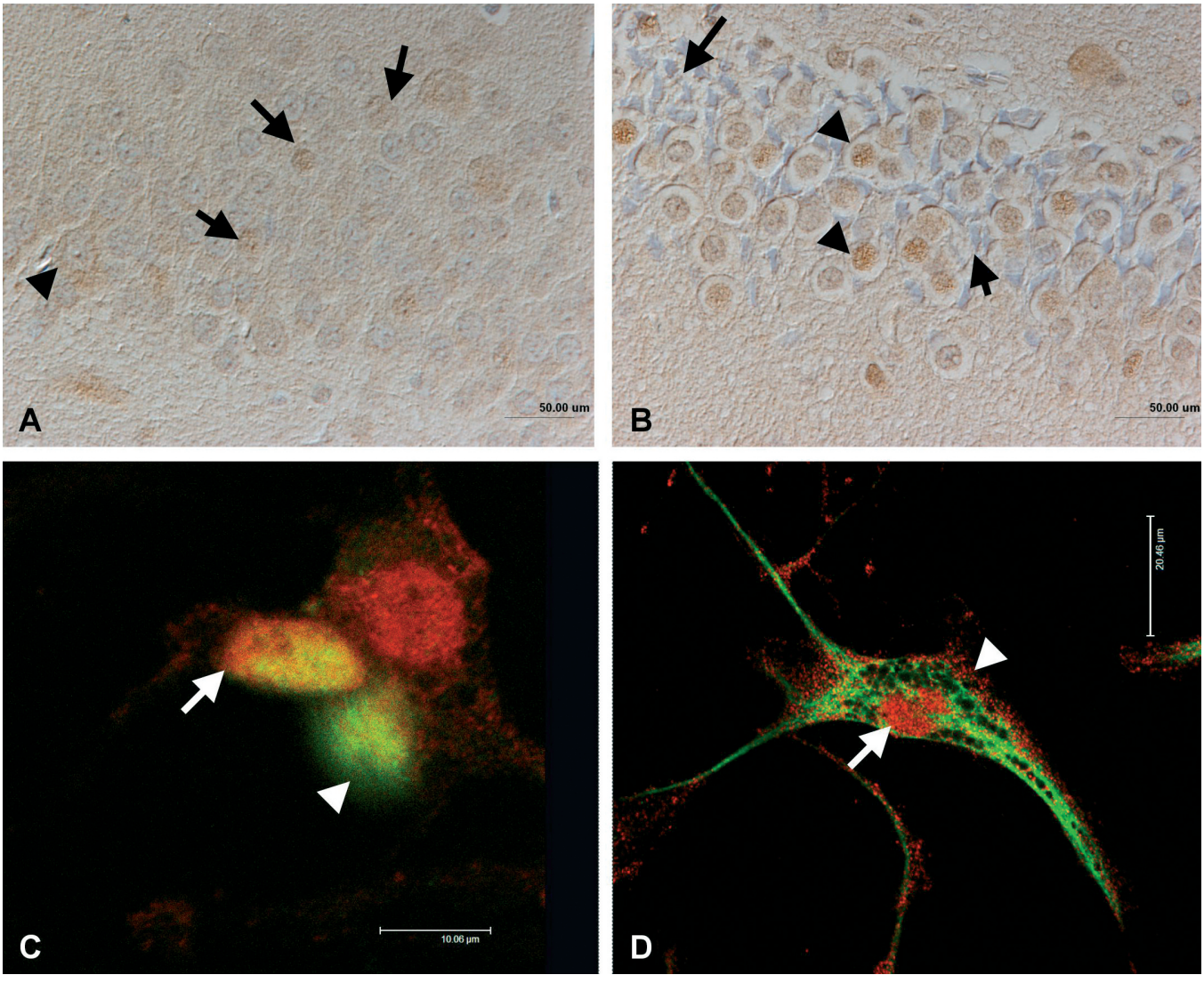

Fig. 3. Subcellular location of ADNP in neurons of adult Sprague Dawley rats. **A, B.** Dentate gyrus. Immunohistological demonstration of ADNP-like immunoreactivity, PAP method, Papanicolaou's haematoxylin counterstain. **A.** Occasional weak expression in nuclei (arrows) and cytoplasm (arrowhead) of granule cells. **B.** Increased nuclear expression (arrowheads) in granule cells in association with multiple dark neurons (arrows). Dark neurons are negative. **C, D.** Immunofluorescence staining of hippocampal dissociated cultures for ADNP (red) and the neuronal marker NeuN (green, C) and GFAP (green, D), respectively. **C.** The majority of neurons exhibit strong nuclear ADNP expression (arrow), with weaker staining in the cell processes. In some neurons, however, ADNP expression is restricted to a relatively weak nuclear reaction (arrowhead). **D.** The majority of astrocytes shows strong nuclear ADNP expression (arrow) and a weaker cytoplasmic reaction (arrowhead).

Smaller cells, most likely representing small neurons in the cerebral cortex, thalamus and hypothalamus, also exhibited a faint to moderate cytoplasmic reaction (Fig. 2C).

Distribution of ADNP positive neurons and fibres

ADNP-like immunoreactivity was observed in all brain areas investigated. In all isocortical regions (orbital, frontal, parietal, occipital, temporal), numerous positive neurons were seen (Fig. 2A,C) and short fibres projecting from the corpus callosum into the deep cortical layer were often positive as well. In the hippocampus, ADNP-like immunoreactivity was restricted to a few pyramid cells in CA1 (Fig. 3A) and CA2 and scattered neurons in the *Stratum oriens* and partly also in the *Stratum radiatum*. In some animals, positive pyramid cells were more numerous in the anterior CA1 region or CA3 region, particularly when scattered dark neurons were present in the CA3 layer. In the polymorph layer of the dentate gyrus (PoDG, formerly called CA4 region) the number of positive cells varied between animals. In the granule cell layer of the dentate gyrus the number of positive cells was generally low; both a cytoplasmic and nuclear reaction was observed. Similarly to the observation made in the CA3 region, more dentate granule cells exhibited a nuclear staining when dark neurons were present in the direct vicinity (Fig. 3B).

Fibres in the basal ganglia (caudate putamen, *globus pallidus*, ventral pallidum, olfactory tubercle, amygdala) were generally ADNP-positive, which rendered the further analysis of cellular ADNP-like immunoreactivity difficult. Fibre tracts of the diagonal band of Broca (VDB, HDB) in the septum were also generally positive. In this area, neurons also displayed a moderate cytoplasmic reaction, whereas other septal nuclei were often negative. In the hypothalamus, some large neurons in the *Nucleus* (N.) *suprachiasmaticus* (SCh) and *N. supraopticus* (SoR) were found to show ADNP-like immunoreactivity. Thalamic ADNP expression was mainly restricted to fibres. In some animals, however, neurons in the *N. reuniens* (Rt) or other thalamic nuclei were positive. In the mesencephalon, pons and medulla oblongata, an intense positive fibre reaction was observed (Fig. 2B). In many brain nuclei, numerous large neurons and motoneurons exhibited an intense granular cytoplasmic reaction (Fig. 2C,D). In the cerebellum, ADNP-like immunoreactivity was generally seen in Purkinje cells and in neurons in the cerebellar nuclei, whereas the numbers/presence of positive cells in the molecular and granular cell layers varied (Fig. 2E). Additionally, in the molecular layer, a few positive fibres were also detected in some animals. Fibres of the cerebellar white matter were generally strongly positive. Results are summarised in Table 1.

Table 1. ADNP expression in different brain areas of adult Sprague Dawley rats.

BRAIN AREA	ADNP IMMUNOSTAINING
Cerebral cortex	++
Hippocampus	+
Septum	+
Basal ganglia	+(+)
Hypothalamus	+(+)
Thalamus	++
Mesencephalon	++
Pons	++
Medulla oblongata	++
Cerebellum	+++

The immunostaining was scored as follows: 0: no ADNP positive cells or staining reactions, (+): very few (up to 5) ADNP positive cells per 400x field and/or very faint staining reactions, +: few (up to 15) ADNP positive cells per 400x field and/or faint stainig reactions, ++: moderate numbers (≥ 15) of ADNP positive cells per 400x field and/or moderate stainig reactions, +++: high numbers (≥ 30) of ADNP positive cells per 400x field and/or strong staining reactions.

ADNP-like immunoreactivity in non-neuronal cells

ADNP-like immunoreactivity was also found in a number of non-neuronal cell types, for example astrocytes. While most astrocytes were ADNP-negative, a few exhibited a cytoplasmic reaction (Fig. 2F). Cell identity was confirmed by the double staining for ADNP and GFAP (Fig. 2F). In the cerebral cortex, some small cells with morphological features of resting microglial cells exhibited cytoplasmic ADNP-like immunoreactivity (Fig. 2C). Due to the generally rather intense fibre reaction in the white matter, oligodendrocytes were difficult to assess for ADNP expression. However, a few perineuronal satellite cells, mainly in the cortex, displayed a cytoplasmic reaction, thereby suggesting that oligodendrocytes can also express ADNP. ADNP-like immunoreactivity was not observed in cells of the choroid plexus. Some ependymal cells, however, exhibited a weak nuclear reaction.

ADNP-like immunoreactivity in dissociated hippocampal cultures

To compare ADNP staining *in vivo* with neurons in primary culture, dissociated hippocampal cultures were analysed. In these cultures, the majority of neurons predominantly exhibited strong nuclear ADNP-like immunoreactivity, with additional weaker staining in the cytoplasm and cell processes. However, occasional neurons only showed very weak nuclear staining (Fig. 3C). The majority of astrocytes in these cultures exhibited strong staining in the nucleus, and additional weaker staining in the cytoplasm (Fig. 3 D).

Discussion

This paper examines the expression of ADNP, a potential neuroprotective protein, in normal rat brain, with the aim of characterising the distribution pattern

and subcellular localisation of ADNP protein within the brain. This is the first systematic evaluation of ADNP expression in the brain, which may help to clarify the function of ADNP in normal and diseased brains. We demonstrate that ADNP is expressed in a wide variety of neuronal and non-neuronal cells, which indicates that ADNP function is not limited to a particular cell type or physiological pathway. Furthermore, we identified ADNP in nucleus and cytoplasm of both neurons and non-neuronal cells. Lastly, we provide evidence that ADNP expression is lost in degenerating neurons, as exemplified by 'dark' neurons, whereas adjacent, morphologically unaltered cells have a more nuclear expression pattern. Predominantly nuclear localisation was also seen in neurons in rat dissociated hippocampal cultures.

Identification of ADNP expressing cell types and brain areas

In this study on adult rat brains, we found that ADNP was widely expressed in the brain parenchyma without obvious individual or gender differences in the regions we examined.

Neurons in many different brain areas expressed ADNP, for example, pyramidal cells, Purkinje cells and motoneurons were frequently ADNP-positive, with the most intense staining being found in motoneurons. Whether this is indicative of a special protective role in motoneuronal homeostasis needs to be determined in future studies. Some non-neuronal cell types, such as astrocytes and microglia and probably also oligodendroglia, were shown to occasionally express ADNP as well, whereas ADNP expression was not found in non-parenchymal cells, such as meningeal cells, choroid plexus cells or endothelial cells.

It should be noted that the ADNP expression described here does not suggest a function in any one specific brain area or physiological pathway. ADNP is known to be essential for accurate brain formation during development (Pinhasov et al., 2003) and the widespread expression in the adult rat brain suggests it also has an important role in later life. This almost ubiquitous ADNP expression might be related to the neuroprotective properties of NAP against so many different stimuli and in so many brain regions.

The main evidence in favour of the hypothesis of ADNP as a neuroprotective molecule comes from the study of the synthetic peptide NAP, which is contained in the ADNP sequence (Bassan et al., 1999; Gozes et al., 1999, 2000). If the wide-ranging protection offered by the peptide NAP is assumed to be reflective of neuroprotective properties of the 'parent' protein ADNP, it would be expected that ADNP is widely expressed in the brain. Indeed, previous studies demonstrated that ADNP was transcribed in the cortex, hippocampus and cerebellum (Bassan et al., 1999; Zamostiano et al., 2001). Our study validates and extends these previous analyses and emphasises the general importance of ADNP throughout the brain.

ADNP expression in astroglial cells

In our study ADNP expression was found most frequently in neurons and occasionally in other cell types such as astrocytes, microglial cells and ependymal cells. This finding supports previous data, where increased levels of ADNP were demonstrated 29 days after closed head-injury, and ADNP expression was localised to microglia and astrocyte-like cells (Gozes et al., 2005). The fact that in our study the majority of astrocytes in the normal rat brain are negative for ADNP, contrasts with this previous finding (Gozes et al., 2005). This may indicate that astrocytes under normal physiological conditions do not constitutively express ADNP, at a level detectable by immunohistology, whereas activated astrocytes, which are seen, for example, after closed head injury (Gozes et al., 2005) may have upregulated or induced ADNP expression. Cultured astrocytes, which we assume to be activated, have previously been shown to express ADNP (Bassan et al., 1999), with localisation in both cytoplasm and nucleus (Furman et al., 2004).

Cellular localisation of ADNP

In positive neurons, ADNP-like immunoreactivity was generally most intense in the perikaryon. The characteristic ADNP immunostaining was a finely granular or coarse reaction product which did not resemble the typical granular synaptic or vesicular expression pattern of secreted proteins such as neuropeptides (Merchenthaler et al., 1993; Nambu et al., 1999). This is consistent with the fact that the ADNP gene does not appear to contain a classical signal peptide for the secretory pathway. Nevertheless, ADNP has been demonstrated in conditioned media harvested from cultured astrocytes; furthermore, levels were enhanced by VIP treatment (Furman et al., 2004). Therefore, ADNP could act in a paracrine fashion and our study does not rule out this possibility.

The predominantly cytoplasmic localisation of ADNP both in the perikaryon and in cell processes is consistent with the previously demonstrated association with microtubules. This association has so far only been shown in astrocytes (Divinski et al., 2004; Furman et al., 2004). Cytoplasmic localisation does not contradict the hypothesis that ADNP can also act in transcription, since some transcription factors, such as NFκB, are sequestered in the cytoplasm when inactive, and only translocate to the nucleus when activated (Baeuerle and Baltimore, 1988). Similarly, the transcription factor Emx2 has been found both in the nucleus and locally in axons, and has been suggested to carry out alternative, non-transcriptional functions when located in the axon, possibly relating to the local translation of protein (Nedelec et al., 2004).

The low number of ADNP-positive nuclei and the generally weak nuclear ADNP-like immunostaining

observed in neurons might well represent transcriptionally active ADNP. In contrast, under *in vitro* conditions (hippocampal dissociated cultures), ADNP expression in neurons was predominantly nuclear, and a less intense reaction was seen in perikaryon and cell processes. This most likely reflects the stresses involved in adaptation to tissue culture. We therefore hypothesise that in the brain, under normal, unchallenged conditions, ADNP is mainly found in the cytoplasm, but in response to a stress or challenge, ADNP localises predominantly to the nucleus. This could explain the differences in ADNP localisation *in situ* and *in vitro*, and also the differences between cytoplasmic and nuclear localisation in different cells of one cell type. Of course, there may be additional cell-type specific distribution.

ADNP and 'dark' neurons

To date, the mechanisms through which different noxious stimuli can influence the activation status of ADNP has not been investigated in detail. It has been previously shown that downregulation (~20%) of ADNP mRNA occurs after treatment with iodoacetate in a model of hypoglycaemic/ischaemic stress (Sigalov et al., 2000). In the study reported here, we propose a similar effect may be occurring, namely in the 'dark' neurons, which are known to occur as a consequence of even very subtle traumatisation of neurons after sistence of the blood supply to the brain parenchyma (Cammermeyer, 1961), but also represent a compromised or degenerate cell population occuring rapidly for example after ischaemia or hypoglycaemia (Csordas et al., 2003) and do not stain for ADNP. Whether the loss of ADNP-like immunoreactivity represents one of the factors leading to a point of no return in cell injury remains speculative. However, the presence of cells with a nuclear ADNP reaction immediately adjacent to the 'dark' neurons might represent a neuroprotective action of ADNP in these cells.

In summary, our study shows that ADNP is expressed by a wide range of neuronal cell types, which is noteworthy since previously ADNP has been considered a predominantly glial protein (Gozes et al., 1999, 2000). Our comparison of the *in vivo* and *in vitro* distribution of ADNP suggests that differential localisation of ADNP correlates with different cell states. Future studies on the regulation of ADNP expression after different brain insults will further clarify the role of ADNP in certain pathological conditions of the CNS, for example epilepsy.

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