Areal and Subcellular Localization of the Ubiquitous Alkaline Phosphatase in the Primate Cerebral Cortex: Evidence for a Role in Neurotransmission

The ubiquitous enzyme TNAP (tissue non-specific alkaline phosphatase) is found in numerous tissues such as liver, kidney and bone, but little attention has been paid to its expression and role in the brain. Observations in TNAP-KO mice, which analyzed the role of this enzyme in osteogenesis, had suggested that TNAP might be involved in GABA neurotransmission. Apart from its presence in endothelial cells, here we show a specific and strong alkaline phosphatase (AP) activity in the neuropile, matching the pattern of thalamo-cortical innervation in layer 4 of the primate sensory cortices (visual, auditory and somatosensory). Such a pattern is also evident in rodents and carnivores, making AP a powerful marker of primary sensory areas. Remarkably, AP activity is regulated by sensory experience as demonstrated by monocular deprivation paradigms in monkeys. The areal and laminar distribution of AP activity matches that of the GAD65, the GABA synthesizing enzyme found in presynaptic terminals. As our electron microscopic investigations indicate that AP is found at the neuronal membranes and in synaptic contacts, it is proposed that the neuronal AP isoform (NAP), may be a key enzyme in regulating neurotransmission and could therefore play an important role in developmental plasticity and activity-dependent cortical functions.

Keywords: GABA, monkey, pyridoxal 5'-phosphate, sensory cortex, TNAP

Introduction

Alkaline phosphatase (AP) consists of a group of iso-enzymes encoded by different gene loci and found in various tissues and cell types. Two of them are devoted to a specific tissue, the placental AP and the intestinal AP (Harris, 1990; Van Hoof and De Broe, 1994). Another isoenzyme, tissue non-specific alkaline phosphatase (TNAP), is expressed under several isoforms in liver, kidney and bone, respectively. AP is also present in endothelial cells of the brain (Hoshi et al., 1997) where it plays a role in the blood–brain barrier. The high endogeneous AP activity in vessels makes it a useful marker to study cerebral vasculature, especially in carnivores and primates (Bannister and Romanul, 1963; Friede, 1966; Manocha, 1970; Bell and Scarrow, 1984; Fonta and Imbert, 2002).

We recently showed that AP is also strongly expressed in the neuropile of the primary visual cortex of marmosets (Fonta and Imbert, 2002), confirming the early observations of Friede (1966) who reported a strong ‘background’ staining of AP in different cortical areas of the macaque. The role of AP in the brain had not been investigated, although ultrastructural and biochemical analyses have shown the presence of this enzyme in neuronal elements including synapses in adult rats (Sugimura and Mizutani, 1979; Zisapel and Haklai, 1980).

The functions of TNAP are mostly investigated in bone where it plays an essential role in osteogenesis. The human syndrome of hypophosphatasia (Henthorn et al., 1992) can be reproduced in TNAP-KO mice by deleting the gene responsible for AP expression (Narisawa et al., 1997). Interestingly, beside effects on skeletal mineralization, TNAP-KO mice develop epileptic seizures whose origin has been linked to deficiency in pyridoxal 5'-phosphate (PLP) and GABA metabolism (Waymire et al., 1995; Narisawa et al., 2001). It was proposed that in TNAP-KO mice, PLP, one of the AP substrates (Fedde and Whyte, 1990), can not enter the intracellular compartment where it functions as a GAD65 cofactor (Martin and Barke, 1998; Soghomonian and Martin, 1998), resulting in a decrease in GABA synthesis which induces changes in the cortical balance between excitation and inhibition.

In the light of this putative role of TNAP in neurons, we investigated the regional expression of AP in the adult marmoset brain at both the light and electron microscopic levels. We found that AP activity was high in layer 4 of the primary sensory areas, with a localization corresponding to the distribution of thalamo-cortical afferents. A remarkable finding of the present study was that AP activity is regulated by sensory experience. The enzyme was localized along the membrane of axonal and dendritic processes in the extracellular side, especially within the synaptic cleft. Altogether, these findings suggest a function for AP in neurotransmission. In addition, a particular areal and laminar correspondence was found between the distribution of AP and GAD65 enzymes. We propose that the neuronal isoform of TNAP, that we have called NAP, is involved in cortical functions and plasticity, probably via a control from thalamo-cortical transmission. Since this NAP isoform, similar to other ubiquitous forms of AP, is conserved in different species (rodent, carnivore and primates), it is possible that this enzyme has an elementary role in the brain.

Material and Methods

Alkaline phosphatase activity was analyzed in the cortex of 10 marmosets (Callithrix jacchus) and three adult macaque monkeys (Macaca mulatta – see Table 1) from the rearing facilities of the Centre de Recherches Cerveau et Cognition. Two of the marmosets were 3 months old (81 postnatal days (PND)), one was 2 months old (PND61), the others were adults. Complementary results obtained from one adult cat and three rats (one adult and two juveniles of 2 and 4 weeks of age) are also presented to analyze similarities of expression across species. AP activity was also examined in an adult rabbit brain. We did not observe significant differences in the results according to the age of the animals used in this study. The early postnatal development of AP expression in the monkey will be the focus of a separate study (Barone et al., 2002; C. Fonta and P. Barone, in preparation).

The procedures used follow national and EEC regulations concerning animal experiments and were approved by the authorized national and veterinary agencies.
The case used in the EM study.

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(Stryker and Harris, 1986). Once the animal was anesthetized with ketamine (25 mg/kg), 10 channels and, consequently, the emission of retinal action potentials was preserved on the nasal end of the whole eyeball was set with a drop of comfort gel and secured by an opaque lens (Dencott Laboratories, France) which covered the mean of a lid suture. The upper and lower eyelids were juxtaposed and sutured. A small opening was preserved for a period of 14 days at PND67 until perfusion at PND81.

In one macaque the monocular deprivation was performed by means of tetrodotoxin (TTX) intracocular injection, which blocks Na channels and, consequently, the emission of retinal action potentials (Stryker and Harris, 1986). Once the animal was anesthetized with ketamine (25 mg/kg), 10 µl of a TTX solution (1.6 µg/µl) was injected with an insulin syringe (29G) into the superior part of the corneal chamber, 1 mm away from the cornea. The animal was killed after surviving 5 days following the single injection, a period during which no sign of pupillary reflex was observed in the injected eye.

**Monocular Deprivation**

In the two 3-month-old marmosets, a monocular visual deprivation was performed for a period of 14 days at PND67 until perfusion at PND81. Deprivation was carried out under general anesthesia (Fonta et al., 1997, 2000a) by means of a 1.5 mixture of xyazine/ketamine (5 mg/kg and 25 mg/kg, respectively) and local anesthesia (xylocaine).

Monocularly deprived animals.

**Monocularly deprived animals.**

**Perfusion and Tissue Processing**

Animals were given a lethal dose of pentobarbital before being perfused intracardially with 0.9% saline added with 0.1% heparin, followed by 4% paraformaldehyde in phosphate buffer (PB) pH 7.4. Brains were immediately removed and put in sucrose solutions of increasing concentrations (10, 20 and 30%) for cryoprotection. Parasagittal or coronal sections (40 or 30 µm thick) were performed on a freezing microtome. In two marmosets and two macaques, the visual cortex was separated from the rest of the brain, physically flattened by 4% paraformaldehyde/0.5% glutaraldehyde mixture in PB pH 7.4. The brain was removed and cut into 50 µm thick sections on a vibratome.

**AP Histochemistry**

We used different methods to reveal AP activity. For light microscopy, endogenous AP reactions were performed by incubating sections in 100 mM Tris–HCl solution (pH 9.5) containing 100 mM NaCl, 50 mM MgCl2, 0.53 mM 4-nitro blue tetrazolium chloride (NBT) and 0.38 mM 5-bromo-4-chloro-3-indolyl-phosphate 4-toluidine salt (BCIP). The reaction was carried out for 10–30 min at room temperature and stopped by transferring sections to 10 mM Tris solution (pH 7.5) with 1 mM EDTA and 10 mM levamisole. Sections were rinsed in Tris and mounted.

For confocal analysis, AP activity was revealed using fluorescence-based techniques with ElF-97 or Fast Red TR as substrates (Cox and Singer, 1999). Sections were incubated for 30–60 min in a solution containing 2.5 mg of naphthol-ASMX-phosphate diluted in 0.5 ml of N,N-dimethylformamide and 5 mg of Fast Red TR salt and adjusted to 5 ml with Tris–HCl buffer (0.1 M) at pH 9.2.

For ultrastructural studies, AP reaction was performed using a modified method of Mayahara et al. (1967). Floating sections were incubated for 30–60 min in a solution composed of 1.4 ml Tris–HCl buffer (0.2 M, pH 8.5), 2.0 ml of sodium β-glycerophosphate (0.1 M), 2.0 ml of magnesium sulfate (15 mM) and 4.0 ml of a saturated lead citrate solution (pH 10.0) and adjusted to pH 9.4 with 0.1 M NaOH. Sections reacted with lead citrate were washed thoroughly in PB, osmicated, dehydrated in ascending series of ethanol and flat embedded in Durcupan ACM (Fluka, Buchs, Switzerland). Ultrathin sections cut from middle, infra- and supragranular layers of V1 of the marmoset were post-stained with lead citrate and examined with a JEOL JEM-1200EX electron microscope.

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**Table 1**

Experimental cases and histo- and immuno-histochemical procedures

<table>
<thead>
<tr>
<th>Experimental cases</th>
<th>Species</th>
<th>Age</th>
<th>AP</th>
<th>CO</th>
<th>Nissl/Neun</th>
<th>GAD65</th>
<th>Myelin</th>
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The numbers of sections proceeded are indicated in each case.

aThe case used in the EM study.

bMonocularly deprived animals.

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as shown in New and Old World monkeys in the visual, auditory and somatosensory systems (Luethke et al., 1989; Krubitzer and Kaas, 1990; Spatz et al., 1994; Barone et al., 2000; Lewis and Van Essen, 2000; Huffman and Krubitzer, 2001b; Lyon and Kaas, 2001). For EM analysis of AP activity, one juvenile marmoset (PND61) was perfused with 0.9% saline added with 0.1% heparin, followed by a 4% paraformaldehyde/0.5% glutaraldehyde mixture in PB pH 7.4. The brain was removed and cut into 50 µm thick sections on a vibratome.
**GAD65 Immunohistochemistry**

In three marmosets, GAD65 was localized using classical immunohistochemistry. Floating sections were incubated for 1 h at room temperature in a blocking solution (10% normal horse serum (NHS) and 0.3% Triton in PBS) then transferred to the primary antibody solution (Boehringer mouse anti-GAD65, 1/2000–1/4000) containing 1% NHS and 0.3% Triton in PBS and incubated for 72 h at 4 °C. Revelation was performed either following standard DAB procedures using the Dako StreptABC Complex Kit or using a streptavidin–FITC-conjugated secondary antibody.

**Microscopy**

Sections were observed in light or fluorescent microscopy with a Leica microscope (DMR) equipped with a CCD camera. Images were acquired using a software (Mosaic, Explora Nova®) installed in a computer directly linked to the microscope stage. Fluorescent confocal images were collected on a Leica microscope (SP2). AP detection using the azo-dye technique was visualized with helium–neon laser stimulation (543 nm) and GAD65 fluorescent immunohistochemistry was visualized with argon–krypton laser stimulation (488 nm).

**Results**

**Specificity of Alkaline Phosphatase Histochemistry**

Histological revelation resulted in two main loci of AP activity in the brain of the marmoset: in the blood vessels and in the neuropile (Fonta and Imbert, 2002). AP labeling in the endothelial cells allows visualization of the dense network of capillaries in the cortical mantle and subcortical structures — see Figure 1 (Fonta and Imbert, 2002).

For example, the architectonic limits of some thalamic nuclei or the presence of the cortical layer 4 (Figs 1B and 2A) can be assessed with their characteristic high density of blood vessels labeled with AP histochemistry. The specificity of labeling is supported by our EM observations of the cellular and ultrastructural localization of AP activity in capillaries (Fig. 1C). In agreement with previous studies, our findings revealed that a strong labeling was associated with the luminal and abluminal surfaces of endothelial cells in the wall of blood vessels (Bannister and Romanul, 1963; Bell and Scarrow, 1984; Bell and Ball, 1985; Lawrenson et al., 1999). As AP belongs to the family of ecto-phosphatases this localization is consistent with its role in the blood–brain barrier, by controlling the transmembrane transport of specific proteins and/or ions and their absorption in the brain (Calhau et al., 1999, 2002).

We tested the specificity of AP labeling in the cortical neuropile first by varying the pH of the incubation solution (Fig. 2). The classical histochemical method used to reveal AP activity is performed at pH 9.5, which corresponds to the optimal level described in biochemical assays of AP activity on different substrates such as PLP or phosphoethanolamine (Bishayee and Bachhawat, 1972; Fedde and Whyte, 1990).

At acidic pH (pH 4.3, Fig. 2D) activity could not be detected, either in the neuropile or in the blood vessels. At neutral pH (7.5), AP activity was nearly absent in the cortical gray matter (Fig. 2B) while a weak labeling remained in endothelial cells (Fig. 2C). Adding AP inhibitors (levamisol and EDTA; Fig. 2E) or omitting the substrate (Fig. 2F) resulted in a complete suppression of AP activity. All these control experiments suggest that the labeling obtained in the neuropile results specifically from the enzymatic activity of AP.

The fact that TNAP is present in diverse isoforms is in agreement with some of our findings. In the rabbit, where blood vessels do not express AP in the brain (Shimizu, 1950), a strong activity was found in the neuropile (data not shown). In the rat, by using Elf-07 as a fluorogenic substrate to detect AP activity (Cox and Singer, 1999), we have not been able to localize AP activity in the brain, although epithelial cells of the intestine as well as the wall of the renal tubules were obviously stained similarly as reported in classical studies (Hoshi et al., 1997). This suggests that the AP activity in the neuropile could derive from a particular isoform, with specific physicochemical properties, conferring on it a different range of affinities for these substrates. Following these observations we propose to use the term of ‘neuronal alkaline phosphatase’ (NAP) in order to distinguish it from TNAP in general.

**Neuronal Localization of AP Activity in the Marmoset Brain**

**Light Microscopy**

In addition to the intense vascular labeling in cortical and subcortical structures in the CNS of marmoset (Fig. 1), histochemical detection of AP activity results in a characteristic laminar pattern within the cortical areas (Fig. 3). There was no evidence of AP activity in the neuropile of subcortical structures including the thalamus, striatum, superior colliculus and hippocampal formation (Figs 1B and 3B–C).

Qualitatively, the intensity of labeling in the neuropile varied across areas. Four main regions exhibited strong NAP expression (Fig. 3A) encompassing (i) the occipital lobe including the primary visual area (V1; Fig. 3B), (ii) the area of posterior bank of the lateral sulcus (LaS) where the primary auditory cortex is located (AI, Fig. 3C), (iii) the somatosensory cortex extending dorsally from the LaS to the interhemispheric wall (Fig. 3B,C) and (iv) the most frontal cortical areas (Fig. 3B). On the other hand, extrastriate visual areas located either in dorsal or ventral parts of the brain were characterized by a low level of NAP activity. Between regions of low and high level of NAP activity, the parieto-dorsal cortex, the inferotemporal lobe and the prefrontal cortex exhibited an intermediate level of staining. Although some areas such as AI, SI or V1 could be identified and delimited by the high level of NAP expression, a more gradual change of NAP activity was found across successive cortical areas. The strongest NAP activity was localized to the middle layers corresponding to granular layer 4 and was confined to the neuropile. No labeling was observed in the perikarya.

**Electron Microscopy**

Previous electron microscopic (EM) studies in the rat cerebral cortex have shown that AP is located on the plasma membrane of nerve cells and in synapses with various combinations of pre- and postsynaptic involvement (Sugimura and Mizutani, 1979; Mori and Nagano, 1985). Our EM examination in area V1 revealed a particularly dense accumulation of metal particles representing NAP activity around neuronal processes in a way that it formed a meshwork outlining the structural elements of the neuropile in layer 4C (Fig. 4B). A similar but much lower density of staining could also be demonstrated in upper and lower layers of V1 (Fig. 4a,c). This is in agreement with the light microscopy observations of a sub-threshold detection of NAP activity in infra- and supragranular layers adding further proof to the specificity of labeling. It should be noted that the labeling sometimes appeared as large grains scattered all over the neuropile in supra- and infragranular layers, which was in
contrast to the granular layer where the same pattern of labeling was observed consistently. As there was no sign of specificity in the subcellular localization of these large grains, it is suggested that it was an artifact of the histochemical procedure.

In AP-positive structures, particles were associated with the extracellular surface resulting in labeling in the tight extracellular space between closely packed neuronal processes as it can be seen on higher power micrographs (Fig. 5b–e). These results are in agreement with the biochemical evidences showing AP as an ecto-enzyme anchored in the plasma membrane (Low and Saltiel, 1988). Strong precipitation was also observed at synaptic junctions (Fig. 5), which is clearly demonstrated in Figure 5a showing a tangential section through the synaptic cleft (arrows). Synaptic contacts of both type I, excitatory-like (Fig. 5a,e,f) and type II, inhibitory-like (Fig. 5b–d) exhibited NAP reactivity as it is identified by the marked difference in the thickening of the postsynaptic densi-

Figure 1. Alkaline phosphatase activity in endothelial cells. (A) High power view of AP expression in the ventral extrastriate visual areas of the marmoset. AP is present in the endothelial cells and enables visualization of the dense network of cortical vasculization. (B) In the visual (LGN) and auditory (MGB) thalamic relays, distinguished by means of CO activity (upper panel), AP, as in the cortex, is present in blood vessels. As seen on a higher magnification of the LGN, there is no evidence of AP activity in the neuropile. Scale bars: 0.1 mm (A); 0.5 mm (B). (C) Electron photomicrograph of a cross-section of a microvessel in the middle layers of area V1 from the very surface of the section. Strong AP-related activity is observed on the luminal surface of an endothelial cell but metal particles are also abundantly present at the abluminal surface (arrows). Arrowheads indicate nuclear/cytoplasmic border. Precipitation of reagent above the heterochromatin is probably an aspecific reaction (see text).

Figure 2. Experimental control of AP specificity. In standard alkaline conditions (pH 9.5) AP expression is present in endothelial cells of blood vessels and in the neuropile of the visual area V1 of the marmoset (A). At neutral (pH = 7.5, panel B, enlarged in C) or acidic pH (pH = 4.3, panel D) AP activity in the neuropile is abolished (but weakly present in the blood vessels at pH = 7.5, panel C). Similarly, adding enzymatic inhibitors (EDTA 1 mM and levamisol 10 mM, panel E) or omitting the substrate (BCIP, panel F) in the incubation solution results in the complete absence of AP activity. Dashed line: V1/V2 border. Scale bars: 1 mm (A, B, D, E); 0.25 mm (C).
ties. In addition, Figure 5b,c show axosomatic contacts which together with the ultrastructural appearance are characteristic features of GABAergic terminals.

We did not observe labeling associated with glial elements while looking at AP activity around glial cell bodies. However, it should be noted that it is technically very difficult, if not impossible, to identify glial processes without using specific markers in our material.

Intracellular labeling was observed mainly over the heterochromatic substance of nuclei within cell bodies (not shown). It should also be noted that a noticeably weaker intracellular labeling was present in the form of small, light granules when compared to the extracellular one (e.g. Fig. 5c–f).

**NAP Expression in Cortical Sensory Areas**

**Primate Studies**

As mentioned above, primary sensory areas exhibit distinctive NAP activity, which is restricted to the granular layers. This localization is reminiscent of those of different histological markers used to identify functional compartments in these areas. Accordingly, a comparative analysis was performed using cytochrome oxidase (CO), acetylcholinesterase (Ache), myelin or Nissl staining to identify the regions of strong NAP activity to specific cortical areas and functional modules. Results of these studies are presented according to the specific cortical areas as follows.

**Visual Cortical Areas.** On flattened visual cortex, when adjacent sections are processed for AP and CO staining (Fig. 6A), the pattern of NAP labeling closely matches that of CO: area V1 is unique as it presents a remarkably high NAP activity in layer 4C which decreases abruptly at the junction with area V2, similar to that of CO labeling (see also Fig. 6B). On parasagittal sections, NAP labeling is nearly undetectable in extrastriate visual areas V2 and V3 (Figs 3B and 6B). Despite this low level of activity, on tangential sections (Fig. 6A) a clear stripe-like pattern of NAP labeling emerged, which was in correspondence with CO stripes (Krubitzer and Kaas, 1990; Rosa et al., 1997; Lyon and Kaas, 2001) in both V2 and V3. However, in supragranular layers of V1, NAP does not delimitate the blobs observed with CO staining (Fig. 6A). In higher-level visual areas, the intensity of expression of NAP increases progressively when moving anterior toward the parietal or inferotemporal areas (see Fig. 3). In contrast to V1, in these regions NAP does not mark clear delineations corresponding to areal boundaries, except for area DM as shown on tangential sections (Fig. 6D).

In V1, a similar strong CO and NAP activity is present in layer 4A. However, while CO activity is uniform across layer 4C, NAP exhibits a characteristic bilaminar pattern of expression, being high in the upper part in layer 4Cα and the lower part in layer 4Cβ, with a clef of weak activity in between (Fig. 6C). In summary, NAP expression exhibits a remarkable compart-
NAP was also strongly expressed in a region anterior and dorsal to the LaS in the anterior parietal cortex, forming a band of high level activity extending medially to the inter-hemispheric wall (Figs 3A,B and 7B). The position of this NAP-positive cortical band corresponded to the localization of somatosensory areas (Huffman and Krubitzer, 2001b; Qi et al., 2002). This identification was confirmed by the presence of strong CO staining that closely matched the NAP-labeling (Fig. 7B, right). Furthermore, Nissl staining showed that the region with high levels of NAP activity had a well defined layer 4 (Fig. 7B, left) which ended anteriorly, at the same level as where NAP activity declined. More anterior from this point, the cortex was characterized by the lack of a layer 4 and the presence of AchE positive Betz cells, which are characteristic features of the motor cortex (Carlson et al., 1986). This region was also characterized by a decrease in AP activity.

**Species Comparisons**

The cat primary visual cortex exhibits striking similarities to the V1 of primates in several aspects, making it an excellent subject for interspecies comparisons. Similarly to the marmoset, especially strong NAP activity was found in layer 4 of the occipital lobe containing visual cortical areas (Fig. 8A,B). However, while in the marmoset only area V1 showed a high level of activity, in the cat NAP was strongly expressed both in areas 17 and 18, as identified by CO histochemistry (Fig. 8A) (Price, 1985), both of which receive afferents from the LGN (Geisert, 1980). NAP labeling sharply decreased at the border between A17 and splenial visual area (SVA) and at the A18/A19 frontier.

NAP activity was also analyzed in the somatosensory cortex of rats and was shown to be highly distributed in layer 4 on coronal sections (Fig. 9A,B). In addition, the pattern of NAP activity formed an identical pattern to that of CO-activity (Fig. 9D), clearly demarcating the individual barrels (Fig. 9B, arrows). This was particularly obvious on sections cut parallel to the cortical surface and passing through layer 4 (Fig. 9C), where NAP expression revealed the representation of individual vibrissae forming the barrel field.

In conclusion, our observations in primates, carnivores and rodents suggest that remarkably high NAP activity is found in layers of sensory cortical areas targeted by afferents from their primary order thalamic nuclei.

**Activity Dependence of NAP Expression**

The high NAP activity in the thalamic recipient layer of primary sensory areas suggested to us that its level could be dependent on afferent activity conveyed by the thalamo-cortical pathways similarly to what is known for CO activity. To test this hypothesis a single dose of TTX was injected in one eye of a macaque monkey. Five days following the injection, on parasagittal sections, NAP activity in layer 4C of V1 showed a patchy organization (Fig. 10A). On flattened sections cut parallel to the cortical layers, NAP bands show alternating pattern of low and high activity in layer 4C (Fig. 10B,C). Adjacent sections reacted for CO present a very similar pattern of labeling (Fig. 10B,C, left), corresponding to ocular dominance columns of injected and intact eyes, respectively, as previously demonstrated (Horton and Hocking, 1998). Using blood vessels as landmarks, we demonstrated that regions of high CO and NAP activity match precisely (Fig. 10B, right) indicating that NAP

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**Figure 4.** AP reactivity at the ultrastructural level in V1. Dense lead citrate precipitation representing NAP activity can be seen in the extracellular space around the neuronal processes in the neuropil in the granular layer (b). Note that synaptic junctions can be easily identified by NAP activity showing the strongest accumulation of metal particles in the granular layer (b). A significantly weaker labeling is present in the supragranular (a) and infragranular (c) layers. Scale bar: 500 nm (applies also to a, b).
activity was diminished in ocular dominance stripes associated with the injected eye.

The drawback of TTX-application is that it can have unwanted effects, such as reducing the anterograde transport of glycoproteins (Riccio and Matthews, 1985). In order to test if the regulation of NAP activity was effectively affected by the level of afferent activity, a 2 week monocular deprivation was performed by means of opaque lens and eyelid suture in juvenile (PND67) marmosets (Fonta et al., 2000a). The deprivation results in a columnar distribution of NAP staining in layer 4C of area V1 (Fig. 10D), similar to that observed after application of TTX in the macaque. Furthermore, decreased NAP activity is registered in layers 4Cα and 4Cβ, supporting its specific relationship with the deprived eye. Notably, the pattern of CO activity is not affected by this short deprivation (Fonta et al., 1997), which suggests that NAP expression is more sensitive than CO to perturbations of incoming activity to the cortex.

**Comparison of the Distribution of NAP Activity and GAD65 Expression**

Lack of TNAP activity in transgenic mice results in an abnormally low level of GABA in the brain as well as reduction of intracellular PLP (Waymire et al., 1995) a GAD65 co-factor (Erlander et al., 1991) suggesting a functional relationship between NAP and GAD65.

We investigated their respective distribution in the marmoset by single fluorescent staining in confocal microscopy. GAD65 exhibits a high level of expression in layer 4C of area V1 (Fig. 11A). High power view of GAD65 labeling, apart from a few cell bodies, is mainly organized in punctate structures (Fig. 12B) that correspond to inhibitory presynaptic terminals (Kaufman et al., 1991; Esclapez et al., 1994; Silver and Stryker, 2000).

There was a striking similarity between the distribution of GAD65ir and NAP activity as both showed an abrupt reduction
Figure 6. NAP activity in the visual areas of the marmoset in three different animals. (A, B) CO (left) and NAP (right) activity on adjacent sections of flattened cortex (A) or on parasagittal sections (B). The pattern of CO staining allows delimitation of visual areas: area V1 shows high CO activity in layer 4C (A, B) and a blob pattern in upper layers (arrowhead in A). Characteristic striped pattern of CO activity (A, arrows) is observed in areas V2 and V3. Dorsal area DM (•) is defined by a high CO level. Level of NAP expression matches with CO activity: high level in layer 4C of area V1 (A, B) and stripes of high activity in areas V2/V3. Note that the level of NAP expression in V1 is far higher than that observed in other extrastriate areas. (C) Higher power view of NAP expression in frontal section of area V1 (see inset on the left for the cortical location) showing that the high level of activity is restricted to layers 4Cα and β while the middle part of 4C presents a weaker level of staining. A moderate level of activity is also present in layer 4A. Scale bars: 5 mm (A); 0.5 mm (B, C).
at the edge of area V1 and a low level in extrastriate areas. Both the somatosensory and the auditory cortex expressed a high level of GAD65 in layer 4 very similar to the distribution of NAP activity (Fig. 11B,C). Examination of adjacent sections reacted for GAD65 and NAP revealed a close correspondence between the localization of these two proteins. This was well illustrated in area V1 (Fig. 12C,D) indicating a similar laminar distribution of GAD65-ir and NAP activity in layers 4A, 4Cα and β with a reduced activity between the two sublayers of 4C. However, GAD65 expression was more restricted to the bottom of 4Cβ than NAP activity.

Discussion
This study shows that AP, an enzyme on which, until now, attention was mainly restricted to its function in other organs including bone, liver, placenta, intestine and vasculature (Van Hoof and De Broe, 1994), is found in mammalian brain, mainly in sensory cortices. Its association with neuronal membranes and synaptic contacts as well as with activity-dependent functions suggest that it may have an important role in regulating cortical neurotransmission.

Localization of NAP in the Brain Suggests its Dependence on Thalamo-cortical Function

NAP and Thalamo-cortical Projections
The high NAP activity in specific areas of the brain suggests that NAP is linked to the thalamo-cortical connections in all the species examined. First, we found a high level of NAP activity in layer 4 of primary sensory areas (visual, auditory and somatosensory), which is specifically targeted by their principal thalamic nuclei. A strong level of NAP activity was also present in the middle layer of the frontal cortex targeted by afferents from the mediodorsal thalamic nucleus. The specificity of NAP localization in the granular layer has been confirmed by EM analysis in V1 showing that the supra- and infragranular layers

Figure 7. NAP activity in the auditory and somatosensory cortex of the marmoset in three different animals. (A) The primary auditory areas, defined by a high level of cytochrome oxidase activity (right) present a strong expression of NAP in layer 4 (left). (B) Left panel: serial sections of the somatosensory cortex reacted for NAP or stained with cresyl violet. The anterior limit of strong NAP labeling in the somatosensory cortex corresponds to the junction with the motor cortex as defined by a lack of clear granular layer and the presence of Betz cells. Right panel: the somatosensory cortex presents a high level of CO activity in layer 4. On the adjacent section, the region of high NAP expression coincides with high CO level. Arrowheads point towards the same blood vessels in individual sections. Insert shows the sections levels. Scale bars: 0.5 mm.
are characterized by a very low level of NAP activity. Secondly, the comparison of NAP localization in layer 4C between New and Old World monkeys shows that the pattern of NAP labeling matches the sublaminar specificity of termination of LGN efferents in these species (Blasdel and Lund, 1983; Lund et al., 1994; Boyd et al., 2000). Thirdly, our results in the postnatal marmoset (Fonta et al., 2000b; Barone et al., 2002) revealed that the developmental expression of NAP in V1 appears sequentially in 4Cα and β similarly to CO activity. Thus NAP and CO activities follow a parallel temporal maturation in magnocellular and parvocellular compartments which is related to the development of the thalamo-cortical pathway (Kennedy et al., 1985; Spatz et al., 1993; Fonta et al., 1997). Fourthly, by depriving the visual cortex from its peripheral input, we have shown eye-specific changes of NAP activity in ocular dominance stripes corresponding to the pattern of termination of LGN efferents (Fonta et al., 2000a).

The pattern of NAP expression suggests also that this enzyme is related to a specific subset of thalamic efferents. In area V1 of the monkey, a strong level of NAP is associated with layers (4A, 4cα and β) that receive inputs from the magnocellular and parvocellular compartments of the LGN (Blasdel and Lund, 1983). However, the CO blobs in layer 3, which are related to inputs from the koniocellular pathway (Livingstone and Hubel, 1982; Lachica and Casagrande, 1992; Hendry and Yoshioka, 1994; Solomon, 2002), are not revealed with NAP labeling. This lack of NAP activity in blobs is consistent with the data obtained in the cat. Several attempts have pointed out the similarities between the M, P and K pathways of the monkey and the X, Y and W channels of the cat, respectively (Casagrande, 1994). In the cat, the thalamic laminae A and A1 (X/Y channel), project only towards areas A17 and 18 (Geisert, 1980; Bullier et al., 1984) which are characterized by a high NAP expression. The C lamina (W channel) has a supplementary cortical projection to A19 where NAP activity is nearly absent in layer 4. Why NAP is differently expressed in these distinct M, P and K channels remains an issue important to the understanding of the role of AP activity in the cortex. Furthermore, the somatosensory area 3a presents a higher level of NAP compared to the adjacent primary motor cortex. While both areas share inputs

Figure 8. NAP activity in the cat visual cortex. (A) On frontal sections, NAP is strongly expressed in layer 4 of both areas A17 and A18. The pattern of NAP activity matches the CO activity observed on adjacent sections. NAP labeling stops abruptly (arrowheads), laterally at the junction with area 19 and medially at the border with area SVA in the splenial sulcus. A closer view of NAP in areas A17–18 (B) reveals that A17 might show a slightly higher level of activity compared to A18. Note the near absence of NAP expression in layer 4 of visual areas such as PMLS in the lateral sulcus. Insert shows the sections levels. Scale bars: 4 mm (A); 2 mm (B).

Figure 9. NAP activity in the somatosensory cortex of the rat from two animals. On frontal sections (A, B) NAP activity is strongly expressed in layer 4 especially in individual barrels (arrows). On flattened cortex, the representation of each vibrissa constitutes the barrel field (C) seen by means of CO reaction (D). Dotted lines circumscribe the same region in both sections using blood vessels (black circles) as landmarks. Scale bars: 1 mm (A, C, D); 0.5 mm (B).
Figure 10. Role of sensory experience on NAP activity in V1. (A–C) Alkaline phosphatase and cytochrome oxidase activities in layer 4C of area V1 in the macaque following monocular deprivation by TTX intra-ocular injection. On parasagittal section, NAP activity is high on the full thickness of layer 4C and shows a patchy distribution with alternating modules of low (arrowheads) and high activity (A). In B and C, V1 operculum was physically flattened and sections cut parallel to the cortical layers. Ocular dominance columns in layer 4C corresponding to the deprived eye, revealed with CO staining, coincide with regions of low activity level of NAP on adjacent section (see composite drawing in B). Black circles in C represent common blood vessels. In two juvenile marmosets (D) a 2 week long monocular deprivation by eye-lid suture performed during the third postnatal month results in a down-regulation of NAP activity in cortical regions corresponding to the deprived eye (arrowheads). Scale bars: 0.5 mm.

Figure 11. Comparison of Gad65 and NAP distribution on serial sections in sensory cortices of the marmoset. Gad65 expression (upper panels) is specifically expressed in layer 4 of primary sensory areas such as V1 (A), the somatosensory (B) and auditory (C) cortex. On serial sections NAP activity (lower panels) matches at both regional and laminar levels with Gad65 pattern.
from the ventral lateral thalamic nucleus, area 3a receives additional sensory projections from the ventral posterior complex (Huffman and Krubitzer, 2001a). Thus, there appears to be an anatomical and therefore functional selectivity in the association of NAP with the type of thalamic projections.

**Regulation of NAP by Neuronal Activity**

Our EM results showed that synaptic contacts exhibit a particularly strong NAP activity and therefore synaptic activity might play a crucial role in regulating NAP. This regulation could be established via activity-dependent pH changes in the nervous system. Accordingly, it was shown that electrical stimulation of the cortex can induce some modifications in extracellular pH (Chesler and Kaila, 1992; Deitmer and Rose, 1996) which in turn can affect neurotransmitters release including GABA (Sitges and Rodriguez, 1998). Therefore, we assume that thalamo-cortical activity results in pH fluctuations in layer 4 similar to those demonstrated in other systems (Traynelis and Chesler, 2001), which in turn could regulate NAP activity.

**NAP and Neurotransmission**

EM analyses clearly showed that NAP activity is located around the neuronal membranes embedding axons and dendrites and also both the pre- and postsynaptic structures. By analogy with TNAp function in other cell types (Calhau et al., 1999), it could be suggested that NAP is involved in active transport of substances and ions and consequently could regulate both axonal influx and neurotransmission. A functional link between AP and neuronal conduction could also be derived from its specific localization in nodes of Ranvier, excluding the axolemma of myelinated axonal segments (Pinner et al., 1964; Mori and Nagano, 1985) as well as the relationship described between AP or PLP deficiencies and myelin abnormalities (Jardim et al., 1994; Narisawa et al., 2001).

Concerning synaptic transmission, we have shown that there is a precise match in the topographic pattern of GAD<sub>65</sub> and NAP expressions in primary sensory areas that show high levels of both enzymes. This specificity of an areal co-expression is also evident at the laminar level such as a restricted expression in the subdivisions of layer 4C in area V1. Epilepsy, which results from an imbalance between cortical excitatory and inhibitory mechanisms (Bernard et al., 2000; Treiman, 2001), has been observed in GAD<sub>65</sub>-KO mice (Asada et al., 1996; Kash et al., 1997), but also in TNAP-KO mice (Waymire et al., 1995; Narisawa et al., 1997, 2001), in infant pyridoxine-dependent seizures (Lott et al., 1978; Kurlemann et al., 1992; Gospe et al., 1994) and in some severe cases of human hypophosphatasia resulting from TNAP gene mutations (Whyte et al., 1988). As PLP, one of the TNAP substrates (Snell and Khaskell, 1970; Spector and Greenwald, 1978; Whyte et al., 1985; Fedde and Whyte, 1990) acts as a co-factor of GAD<sub>65</sub> (Erlander and Tobin, 1991), which in turn could regulate NAP activity. This regulation could be realized by the same PLP-dependent enzyme, the aromatic amino acid decarboxylase (AADC; Dakshinamurti et al., 1990; Hartvig et al., 1995). Similarly to the pattern of NAP expression, there is a high density of serotonin-containing fibers in sensory regions in primates (Berger et al., 1988; de Lima et al., 1988; Wilson and Molliver, 1991) and especially in layer 4C of area V1 in the marmoset (Hornung et al., 1990). A functional link between NAP and 5HT is plausible since AADC is localized in serotonergic axons, fibers and terminals in the cerebral cortex (Bernard et al., 1991).

**Role of NAP in Activity-dependent Mechanisms**

Variation in cortical AP activity has been observed in chemically induced kindling which affects the GABA system (Erakovic et al., 2001) and, hypothetically, AP would dephosphorylate neurotransmitter receptors. Because of its topographical association with GAD<sub>65</sub>, NAP may be involved in the regulation of GABA synthesis in layer 4 of the cerebral cortex. Among the two GAD isoforms (GAD<sub>65</sub> and GAD<sub>67</sub>) involved in GABA synthesis (Erlander et al., 1991; Soghomonian and Martin, 1998), GAD<sub>65</sub> is related to fast GABA release and regulate both axonal influx and neurotransmission. A functional link between AP and neuronal conduction could also be derived from its specific localization in nodes of Ranvier, excluding the axolemma of myelinated axonal segments (Pinner et al., 1964; Mori and Nagano, 1985) as well as the relationship described between AP or PLP deficiencies and myelin abnormalities (Jardim et al., 1994; Narisawa et al., 2001).
et al., 1998). Because of its presence in the synaptic cleft of type II contacts, we suggest that NAP is directly involved in regulating rapid changes of GABA synthesis in response to alterations in afferent activity. However, the function of NAP localized in Type I synapses resembling glutamatergic contacts remains to be elucidated. Altogether, NAP would be in a good position to insure cortical function and plasticity by regulating neurotransmitter synthesis in the thalamo-recipient layers and making it a key element in mechanisms regulating cortical map reorganizations following partial deafferentation (Jones, 1995; Buonomano and Merzenich, 1998; Jones, 2000). Finally, the presence of NAP in the prefrontal cortex suggests it has a role in higher cognitive functions and probably dysfunctions, via the regulation of the GABA level (Goldman-Rakic and Selemon, 1997; Lewis et al., 1999).

Conclusion

The areal and laminar topography of NAP expression constitutes a characteristic marker for sensory areas as well as the territories of the thalamo-cortical projections. NAP participates in the particularity of local cortical networks (or modules) which, in addition to the pattern of connectivity, confers on them their unique functional properties. The link of NAP to various biochemical functions through PLP, such as neurotransmitter synthesis, regulation of metabolic pathways and synaptic transmission, makes it a crucial element of cortical plasticity.

Notes

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