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REGULAR ARTICLE

Differential expression of the bone and the liver tissue non-specific alkaline phosphatase isoforms in brain tissues

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Abstract The enzyme tissue non-specific alkaline phosphatase (TNAP) belongs to the ectophosphatase family. It is present in large amounts in bone in which it plays a role in mineralization but little is known about its function in other tissues. Arguments are accumulating for its involvement in the brain, in particular in view of the neurological symptoms accompanying human TNAP deficiencies. We have previously shown, by histochemistry, alkaline phosphatase (AP) activity in monkey brain vessels and parenchyma in which AP exhibits specific patterns. Here,

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we clearly attribute this activity to TNAP expression rather than to other APs in primates (human and marmoset) and in rodents (rat and mouse). We have not found any brain-specific transcripts but our data demonstrate that neuronal and endothelial cells exclusively express the bone TNAP transcript in all species tested, except in mouse neurons in which liver TNAP transcripts have also been detected. Moreover, we highlight the developmental regulation of TNAP expression; this also acts during neuronal differentiation. Our study should help to characterize the regulation of the expression of this ectophosphatase in various cell types of the central nervous system.

Keywords Ectophosphatase · Cerebral endothelial cell · Neuron · Primate · Rodent

Introduction

Alkaline phosphatases (APs) constitute a particular group of ectoenzymes that are anchored on the external leaflet of plasma membranes by a glycosyl phosphatidyl inositol (GPI) moiety, thereby conferring the capacity for such phosphatases to act on substrates located in the extracellular space (Millan 2006). These phosphatases are expressed in the brain in which ectophosphatases and ectokinases are thought to be important for cerebral development and function (Ehrlich et al. 1986; el-Moatassim et al. 1992; Plesner 1995; Robson et al. 2006). Although little is known about its particular function, AP activity has been used for many decades as a marker of brain microvessels in primate brain (Anstrom et al. 2002; Bell and Ball 1985; Fonta and Imbert 2002; Moody et al. 2004). In addition to microvessels, the presence of AP has been described in the cerebral parenchyma of young and adult monkeys (Fonta and Imbert 2002; Fonta et al. 2004, 2005). However, most of the data concerning AP expression in nervous tissue relates to enzymatic detection coupled to histological studies. In this context, many phosphatases and nucleotidases exhibiting some overlapping substrate specificities are notably present in the brain (Langer et al. 2007, 2008). Therefore, a clear identification of the APs expressed in cerebral tissues and in specific cell types remains to be undertaken in order to analyse their functions further in the brain.

The APs represent a family of conserved isoenzymes identified in many animal species. In humans, four isoenzymes have been described. The intestinal (IAP), the placental (PLAP) and the germinal (GCAP) APs are expressed in the tissues for which they are named and are encoded by separate homologous gene loci, respectively: *ALPI*, *ALPP*, and *ALPPL*. The tissue non-specific alkaline phosphatase (TNAP: EC 3.1.3.1) is encoded by the *ALPL* gene. TNAP is mainly expressed in bone, liver and kidney but is also found in other tissues. In mouse, five loci have been identified, including the *Akp5* embryonic-specific and the *Akp6* intestinal-specific AP genes, and the *Akp2* tissue non-specific AP-gene. One TNAP gene (*Alp 1*) and two intestinal AP genes have been reported in rat (Millan 2006). Histochemistry or enzymatic tests performed in the presence of levamisol, a potent uncompetitive inhibitor specific of TNAP (Van Belle 1976), have indicated the presence of TNAP in the primate and rodent nervous tissue (Ermonval et al. 2009; Fonta et al. 2004; Langer et al. 2008; Narisawa et al. 1994). However, only a few reports have identified TNAP by using specific antibodies (Ermonval et al. 2009; Langer et al. 2007) and, only recently, TNAP expression has been shown to be induced during bioaminergic neuronal differentiation of a mouse neuroepithelial cell line (Ermonval et al. 2009).

The physiological role of TNAP, which acts as a phospho-monoesterase with a high optimum pH, has yet to be defined in most tissues, with the exception of bone in which its role in mineralization has been clearly established (Hessle et al. 2002; Wennberg et al. 2000). Indeed, in humans, a deficit of this enzyme resulting from mutations in the *ALPL* gene leads to a rare metabolic inherited bone disease known as hypophosphatasia (Rathbun 1948; Whyte 1994). In the central nervous system (CNS), several lines of evidence favour a role of TNAP in brain functions. In particular, clinical observations have associated neurological disorders with abnormal TNAP serum levels. On the one hand, some patients exhibiting severe forms of hypophosphatasia with low serum level of TNAP suffer from epilepsy (Whyte 1994). On the other hand, a form of hyperphosphatasia characterized by an abnormally high level of TNAP activity in the serum is accompanied by mental retardation, seizures and neurological deficits (Kruse et al. 1988; Mabry et al. 1970; Thompson et al. 2010). Interestingly, epileptic symptoms are also reproduced in *Akp2*-/- mice totally deprived of TNAP activity (Narisawa et al. 1997; Waymire et al. 1995). These observations can be linked to recent data indicating a role of TNAP in neurotransmitter metabolism (Balasubramaniam et al. 2010; Ermonval et al. 2009).

In the present study, we demonstrate that AP activity detected in the parenchyma and in endothelial cells of brain from human and other species results from the expression of the *ALPL* gene. In this context, two different TNAP transcripts have notably been identified. The *APLP* gene contains 12 exons, the first exon being part of the 5′-untranslated region (UTR) of the TNAP mRNA (Weiss et al. 1988). This 5′-UTR consists of either exon 1A or exon 1B obtained by alternative transcription initiation. The transcription of the upstream exon 1A is preferentially driven in osteoblasts, whereas transcription is preferentially initiated with exon 1B in liver and kidney (Matsuura et al. 1990; Studer et al. 1991; Toh et al. 1989). Therefore, the

so-called bone and liver TNAP isoforms exhibit the same amino acid sequence but differ at the level of their mRNA whose promoter can be specified according to the exon 1 that is present in the transcript. At present, no data from the literature documents the TNAP transcripts expressed in the CNS. Here, by using the marmoset model, we rule out the presence of any brain-specific promoter and show that the brain AP corresponds to the expression of the *ALPL* gene driven by the bone promoter. However, a remarkable species specificity is highlighted by the finding of an additional transcript starting with exon 1B in mouse neurons. In addition, our study reveals that TNAP expression varies according to the developmental stage. These data should help further investigations of TNAP functions in the brain.

Materials and methods

Tissues and cell culture

Human brain samples were collected by the Human Brain Tissue Bank, Budapest, in accordance with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999) and the Code of Ethics of the World Medical Association (Declaration of Helsinki). Brain samples were taken during brain autopsy and were provided by the former National Institute of Psychiatry and Neurology and the Neuropharmacological and Neuroendocrine Research Laboratory of the Semmelweis University and the Hungarian Academy of Sciences. Written informed consent was obtained from the next of kin. Samples were taken from the prefrontal cortex. They originated from two adults: a 52-year-old man with no neurological or affective disorders (sample used for polymerase chain reaction [PCR] analyses), and a 95-year-old man with no tau pathology, amyloid angiopathy, or any other lesion in the region examined for TNAP. This last sample was used for histology. Parallel histochemistry analyses performed by our group showed no change in cortical AP activity patterns in humans throughout the adult life (Negyessy et al. 2010).

Marmoset (*Callithrix jacchus*) brain samples were obtained from the rearing facilities of the Centre de Recherche Cerveau et Cognition (CerCo, CNRS/Université Paul Sabatier, Toulouse 3, France). For PCR analyses, samples were taken from the grey or white matter of the cerebral hemispheres or from the cerebellum at two different postnatal ages (PND 3 or PND 12) and from 7-year-old adults. Histology was performed on a 2-year-old marmoset. Our previous work had shown that AP activity pattern did not change between 2 and 11 years of age in marmosets (Fonta et al. 2004, 2005). Liver, bone and kidney samples were taken from adult monkeys. All experimental procedures used were in accordance with the recommendations of the EEC (86/609/EEC), the French National Committee and the Regional Committee for the Use of Laboratory Animals.

C57Bl/6 mouse brain and spinal cord samples of adults and embryos at embryonic day 15 (E15) were obtained from the CerCo. Adult and E15 embryo Wistar rat brain samples were from INSERM, UMR S975, Paris, France. Liver samples were taken from adult rat and mouse or from E15 mouse embryo. Intestine samples were isolated from adult mouse. Animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals (National Research Council 1996), European Directive 86/609 and the guidelines of the local institutional animal care and use committee.

Neuronal or endothelial cells were either derived from cell lines or isolated from cerebral tissue. Primary cell cultures of glial cells or neurons were derived from rat embryo brains. Embryos were extracted at day 15.5 from previously euthanized pregnant Wistar rats; the cortex from each embryo was dissected, collected and mechanically triturated. Cell suspensions containing post-mitotic cortical neurons were maintained as previously described (Schmidt et al. 2009). Cell division was prevented by adding 2 μM ARAC (arabinoside C) at DIV1 (day in vitro 1) and 2 μM MK-801 (dizocilpine) was added at DIV1 to avoid excitotoxicity. Cells collected at DIV7 were free of glial and endothelial cells and were thus considered as cortical neurons. For the rat neuron-free cultures, dissociated cortex was plated in flasks (Corning) pre-coated with 1 mg/ml polyethylenimine and cultured in 44% DMEM/44% F12 medium containing 10% fetal bovine serum and antibiotics (Gibco, Invitrogen). Such cells collected at DIV7 mainly consisted of glial cells.

The mouse neuronal differentiation model consisted of the 1C11 neuroepithelial cell line and its fully differentiated neuronal progenies, viz. either serotonergic ($1C11^{5\text{-HT}}$) or noradrenergic ($1C11^{NE}$) progenies, taken respectively at day 4 and day 12, as previously described (Mouillet-Richard et al. 2000).

The human brain immortalized endothelial cells line (hCMEC/D3) representing a well-known in vitro blood brain barrier model were cultured in Petri dishes precoated with rat tail collagen type 1, for 3-4 days (Weksler et al. 2005).

Isolation of primary mouse brain microvascular endothelial cells (pMBMECs) from 4-week-old C57Bl/6 mice was as previously published (Coisne et al. 2005). The isolation protocol resulted in a mixture of erythrocytes and pMBMECs. Erythrocytes were depleted from cultured pMBMECs by two washing steps that removed all non-adherent cells 1 day after the isolation procedure. All pMBMEC procedures were described previously in Lyck et

al. (2009). These cells were immediately processed for RNA isolation or cultured for a few days. Culture processing in this protocol did not impact on cell life but may have altered the relative expression of genes (Lyck et al. 2009).

Histochemical detection of AP activity

AP was detected in free-floating sections (40 μm thick), obtained from tissue fixed in 4% paraformaldehyde, by histochemistry with BCIP (5-bromo-4-chloro-3-indolyl-phosphate-toluidine salt) as substrate and revealed with NBT (4-nitro-blue tetrazolium chloride). The AP activity so detected had been previously shown to be inhibited by the TNAP-specific inhibitor levamisole (Fonta et al. 2004; Langer et al. 2008).

RNA preparation

Total RNA was purified from rodent and primate tissues at various stages and from cell cultures (neuronal and glial rat primary culture and human endothelial cells) by using the High Pure purification kit (Roche). In the case of 1C11, $1C11^{5\text{-HT}}$ and $1C11^{NE}$ cells, the RNeasy midi kit (Qiagen Inc) was used.

RNA from freshly prepared pMBMEC was prepared by using a commercial RNA isolation kit including an on-column DNAse digestion (RNAready, Biodiagnostik; DNAse from Qiagen). The same protocol was applied to the preparation of mRNAs from pMBMEC cultured for several days.

Reverse transcription with PCR and analytical PCR

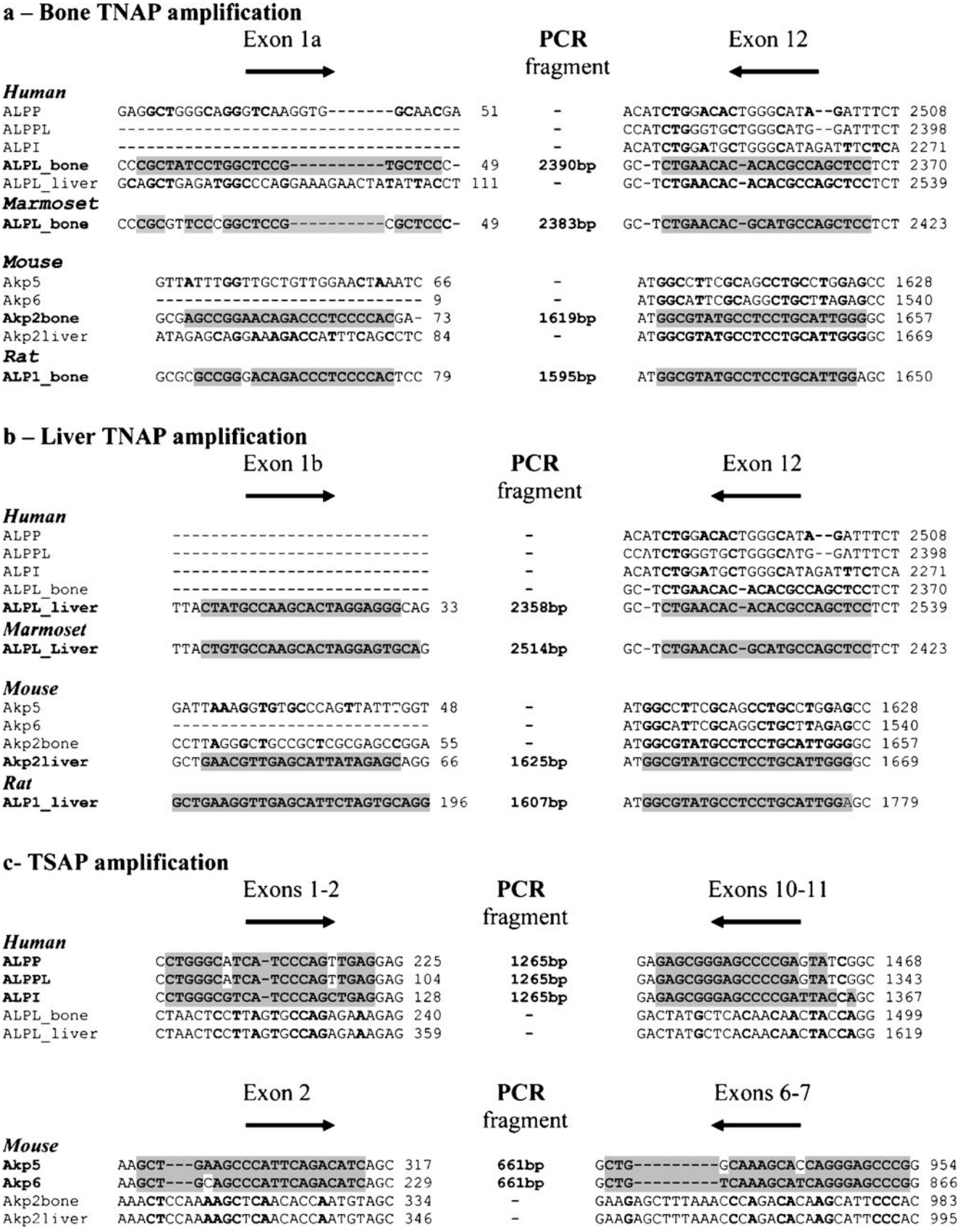
Reverse transcription with PCR (RT-PCR) was performed with the Transcriptor first-strand cDNA synthesis kit (Roche Diagnosis) by using 1 μg total RNA and the poly dT primer, according to the manufacturer's instructions. These cDNAs were then characterized by PCR with various sets of primers specific to the transcript tested according to the animal species (Fig. 1). To detect bone or liver TNAP in primate tissues, primers were chosen in exon 1A and exon 1B (forward) and exon 12 (reverse) of the *ALPL* gene. Tissue-specific AP expression was explored by primers designed in a sequence conserved in the three human tissue-specific APs (*ALPP*, *ALPPL*, *ALPI*). For the marmoset, tissue-specific AP sequences were deduced from the marmoset genome (produced by the Washington University St. Louis [WUSTL] School of Medicine [Genome Sequencing Center] in St. Louis, Mo., USA) by alignment with human tissue-specific AP sequences by using the BLAT aligner from the UCSC genome browser http://genome.ucsc.edu/cgi-bin/hgGateway. For mouse tissue-specific AP *Akp5* and *Akp6*, primers located in conserved sequences of exon 2 (forward primer) and exons 6-7 (reverse primer) were chosen. PCR was performed with the PCR MasterMix kit (Promega) or *Taq* Gold polymerase (Applied Biosystems). Conditions for amplification with the PCR MasterMix were as follow: denaturation at 95°C for 5 min followed by 35 cycles at 95°C for 40 s, 55°C for 40 s and 72°C for 3 min. PCR amplifications with the *Taq* Gold polymerase were carried out in three steps: first step at 95°C for 15 min, followed by a second step of 40 cycles of 30 s at 95°C, 60 s at 55°C or 60°C depending on the primers and 72°C for 2 min 30 s, and a third step of extension at 72°C for 5 min. Amplified fragments were analysed in a 1% agarose gel and sizes were evaluated compared with the migration of standards.

The sizes of the expected fragments were 1265 bp and 661 bp for human/marmoset and mouse tissue-specific AP, respectively. For bone/liver TNAP, the sizes were: 2390/2358 (human), 2383/2514 (marmoset), 1619/1625 (mouse) and 1595/1607 (rat). An additional reverse primer located in exon 12 (see below Fig. 4a) was used to amplify human bone TNAP [5′-TGCAGGCTGCATACGCCATC-3′] with an expected fragment size of 1639 bp.

RACE and sequencing of marmoset TNAP

The 5′ and 3′ cDNA ends were determined by using the SMART RACE (rapid amplification of cDNA ends) cDNA Amplification Kit (Clontech). Briefly, the method incorpo-

Fig. 1 Primers used for specific amplification of primate and rodent tissue-non-specific (TNAP) and tissue-specific (TSAP) alkaline phosphatases (APs). Specificity of the primers and sizes of the expected fragments obtained by the polymerase chain reaction (PCR; PCR fragment column) are illustrated on aligned exons of corresponding AP sequences for both primates and rodents. Each set of primers used to detect the bone TNAP transcripts (**a**), the liver TNAP transcripts (**b**) or the TSAP transcripts (**c**) are highlighted in *grey*. The forward primers designed to detect the two TNAP transcripts in human (*ALPL*_bone and *ALPL*_liver) and mouse (*Akp2*bone and *Akp2*liver) specifically match their respective cDNA sequence, with no cross-homology between them, and do not present any sequence homology with TSAP cDNAs (similar bases are indicated in *bold*). Primers used to detect human or mouse TSAPs (**c**) were designed from *ALPI* and from *Akp5* sequences, respectively. The *ALPI* primers show a quasi complete homology (except for two positions not highlighted on the corresponding sequences) with the corresponding sequences identified in APs from human placenta (*ALPP*) and germ cells (*ALPPL*). The same high homology appears for the mouse embryonic (*Akp5*) and intestinal (*Akp6*) TSAP. These TSAP-specific primers only display a few common nucleotides (indicated in *bold*) with TNAP sequences. Accession numbers of the cDNA sequences used for these alignments are as follows: Human *ALPI*: NM_031313.2, Human *ALPP*: NM_001632.3, Human *ALPPL*: NM_001631.3, Marmoset *ALPL*: GQ465850.1, Mouse *Akp2* (bone): NM_007431, Mouse *Akp2* (liver): NM 007431.2, Mouse *Akp5*: NM_007433, Mouse *Akp6*: NM_001081082, Rat *Alp1*: NM013059. The human *ALPL* cDNA sequence was taken from Weiss et al. (1988) for *ALPL*_bone and from Matsuura et al. (1990) for *ALPL*_liver


a – Bone TNAP amplification
Exon 1a
PCR fragment
Exon 12
Human
ALPP GAGGCTGGGCAGGGTCAAGGTG-------GCAACGA 51 - ACATCTGGACACTGGGCATA--GATTTCT 2508
ALPPL ---------- - CCATCTGGGTGCTGGGCATG--GATTTCT 2398
ALPI ---------- - ACATCTGGATGCTGGGCATAGATTTCTCA 2271
ALPL_bone CCCGCTATCCTGGCTCCG----------TGCTCCC- 49 2390bp GC-TCTGAACAC-ACACGCCAGCTCCTCT 2370
ALPL_liver GCAGCTGAGATGGCCCAGGAAAGAACTATATTACCT 111 - GC-TCTGAACAC-ACACGCCAGCTCCTCT 2539
Marmoset
ALPL_bone CCCGCGTTCCCGGCTCCG----------CGCTCCC- 49 2383bp GC-TCTGAACAC-GCATGCCAGCTCCTCT 2423
Mouse
Akp5 GTTATTTGGTTGCTGTTGGAACTAAATC 66 - ATGGCCTTCGCAGCCTGCCTGGAGCC 1628
Akp6 ---------- 9 - ATGGCATTCGCAGGCTGCTTAGAGCC 1540
Akp2bone GCGAGCCGGAACAGACCCTCCCCACGA- 73 1619bp ATGGCGTATGCCTCCTGCATTGGGGC 1657
Akp2liver ATAGAGCAGGAAAGACCATTTCAGCCTC 84 - ATGGCGTATGCCTCCTGCATTGGGGC 1669
Rat
ALP1_bone GCGCGCCGGGACAGACCCTCCCCACTCC 79 1595bp ATGGCGTATGCCTCCTGCATTGGAGC 1650
b – Liver TNAP amplification
Exon 1b
PCR fragment
Exon 12
Human
ALPP ---------- - ACATCTGGACACTGGGCATA--GATTTCT 2508
ALPPL ---------- - CCATCTGGGTGCTGGGCATG--GATTTCT 2398
ALPI ---------- - ACATCTGGATGCTGGGCATAGATTTCTCA 2271
ALPL_bone ---------- - GC-TCTGAACAC-ACACGCCAGCTCCTCT 2370
ALPL_liver TTACTATGCCAAGCACTAGGAGGGCAG 33 2358bp GC-TCTGAACAC-ACACGCCAGCTCCTCT 2539
Marmoset
ALPL_Liver TTACTGTGCCAAGCACTAGGAGTGCAG 2514bp GC-TCTGAACAC-GCATGCCAGCTCCTCT 2423
Mouse
Akp5 GATTAAAGGTGTGCCCAGTTATTTGGT 48 - ATGGCCTTCGCAGCCTGCCTGGAGCC 1628
Akp6 ---------- - ATGGCATTCGCAGGCTGCTTAGAGCC 1540
Akp2bone CCTTAGGGCTGCCGCTCGCGAGCCGGA 55 - ATGGCGTATGCCTCCTGCATTGGGGC 1657
Akp2liver GCTGAACGTTGAGCATTATAGAGCAGG 66 1625bp ATGGCGTATGCCTCCTGCATTGGGGC 1669
Rat
ALP1_liver GCTGAAGGTTGAGCATTCTAGTGCAGG 196 1607bp ATGGCGTATGCCTCCTGCATTGGAGC 1779
c- TSAP amplification
Exons 1-2
PCR fragment
Exons 10-11
Human
ALPP CCTGGGCATCA-TCCCAGTTGAGGAG 225 1265bp GAGAGCGGGAGCCCCGAGTATCGGC 1468
ALPPL CCTGGGCATCA-TCCCAGTTGAGGAG 104 1265bp GAGAGCGGGAGCCCCGAGTATCGGC 1343
ALPI CCTGGGCGTCA-TCCCAGCTGAGGAG 128 1265bp GAGAGCGGGAGCCCCGATTACCAGC 1367
ALPL_bone CTAACTCCTTAGTGCCAGAGAAAGAG 240 - GACTATGCTCACAACAACTACCAGG 1499
ALPL_liver CTAACTCCTTAGTGCCAGAGAAAGAG 359 - GACTATGCTCACAACAACTACCAGG 1619
Exon 2
PCR fragment
Exons 6-7
Mouse
Akp5 AAGCT---GAAGCCCATTCAGACATCAGC 317 661bp GCTG---------GCAAAGCACCAGGGAGCCCGG 954
Akp6 AAGCT---GCAGCCCATTCAGACATCAGC 229 661bp GCTG---------TCAAAGCATCAGGGAGCCCGG 866
Akp2bone AAACTCCAAAAGCTCAACACCAATGTAGC 334 - GAAGAGCTTTAAACCCAGACACAAGCATTCCCAC 983
Akp2liver AAACTCCAAAAGCTCAACACCAATGTAGC 346 - GAAGAGCTTTAAACCCAGACACAAGCATTCCCAC 995

rated synthetic adaptors into both the 5′ and 3′ ends of cDNA during synthesis, thereby allowing the cloning and the sequencing of the complete cDNA sequence without having to presume the 5′ and 3′ extremities of the mRNA. The two TNAP gene-specific primers were chosen in a region of the *ALPL* gene conserved among species, ALPL-GSP2-3′RACE (5′-GGGAGTGGTCCGCGGTGACCA CAGTC-3′) for the 3′ reaction and ALPL-GSP1-5′RACE (5′-CTGGACGGCCTGGACCTCGTCGACATC-3′) for the 5′ reaction.

The complete marmoset brain TNAP cDNA was then sequenced with the primers normally used to sequence the human *ALPL* cDNA. PCR products were directly sequenced by using the ABI PRISM Dye Terminator Reaction kit (Applied Biosystems).

Results

TNAP is expressed in adult brain of various mammalian species

Our previous work has revealed strong AP activity in monkey nervous tissues not only at the level of the microvessels, but also in neurons (Fonta et al. 2004). In the present study, brain AP characterization was pursued and histochemistry was performed on brain sections from primates, including human prefrontal cortex and whole marmoset brain, and from rodents, including whole rat or mouse brains (Fig. 2). AP activity was found in both rodent and primate brain parenchyma. It most probably corresponded to AP activity at the neuronal cell surface, electron-microscopical analyses having previously detected it lining the outer surface of the plasma membrane of neuronal cells (Fonta et al. 2004). Under our experimental histochemical conditions, AP activity was not detected inside cell bodies. Interestingly, different AP patterns were displayed in primate and rodent adult brains. Namely, a laminar pattern was observed in human (Fig. 2a) and marmoset (Fig. 2b) cerebral cortex as previously described (Fonta et al. 2004). By contrast, AP staining of rodent brain exhibited various levels of intensity including many cortical and sub-cortical regions (Fig. 2c, d) similar to data obtained in mouse by Langer and collaborators (2008). Moreover, we observed a higher level of AP activity in the superior layers of various cortical regions (retrosplenial, somatosensory and piriform cortices), in the hypothalamus and in the amygdala. In the thalamic nuclei and habenula, AP activity appeared to be weaker, especially in the rat. In all species, the white matter (rich in myelinated fibers) and fibre tracts (e.g. corpus callosum, fimbria, fornix, mammillo-thalamic tract) showed no obvious AP staining at light-microscopic resolution. As illustrated in the insets of Fig. 2, AP staining at a higher magnification appeared to be prominent in the blood vessel walls of each species investigated. It corresponded to AP activity at the level of the endothelial cell membrane, as previously shown by electron-microscopical investigation (Fonta et al. 2004). Previous data obtained by using levamisole at a basic pH showed the total inhibition of AP activity in primates (Fonta et al. 2004; Negyessy et al. 2010) and in murine brain tissue or neuronal cell cultures (Ermonval et al. 2009; Langer et al. 2008) suggesting that brain AP was attributable to TNAP expression. Here, we performed PCR analyses to eliminate possible involvement of tissue-specific APs. We did not detect any amplification by using the tissue-specific AP primers, either in marmoset (Fig. 2e, lanes 3-5) or human (Fig. 2e, lane 10) brain. However, a signal corresponding to the size of the IAP was obtained in the kidney sample, known to express TNAP activity (Nouwen and De Broe 1994). This illustrated the efficiency and specificity of the primers used and showed that the absence of signal in the brain was significant. We also demonstrated that the tissue-specific APs were not expressed in mouse embryo nervous system or adult brain (Fig. 2f, lanes 1, 2, 8).

These results ruled out tissue-specific APs being expressed in adult cerebral tissue, while supporting the hypothesis that AP activity in different brain areas corresponded to the TNAP.

No brain-specific TNAP promoter is used in adult cerebral tissues

Since AP activity was strongly suggested to result from TNAP expression in the brain, we next sought to identify the *ALPL* promoter used for brain expression. We used our marmoset model to characterize TNAP transcripts by using a primer extension procedure (RACE analysis). TNAP-specific primers were designed in a well-conserved region coding for the TNAP catalytic domain. This allowed us to amplify a single band, both for the 5′ and the 3′ end reaction. Then, the sequencing of the PCR fragments led to a determination of the complete cDNA sequence of the marmoset TNAP, which was at that time unknown (our GenBank submission number GQ465850). This nucleotide sequence showed 90.2% homology with human *ALPL* and predicted that the marmoset TNAP protein had 96% identity with human TNAP (Fig. 3a). The twenty amino-acid residues differing between marmoset and human TNAP were positioned on the three-dimensional human TNAP model (Fig. 3b) proposed by Mornet and collaborators (2001). We found 18 of these amino-acids to be located in three of the five functional domains of the protein: (1) in the hydrophobic end that is cleaved off and replaced by the GPI-anchor and that therefore does not

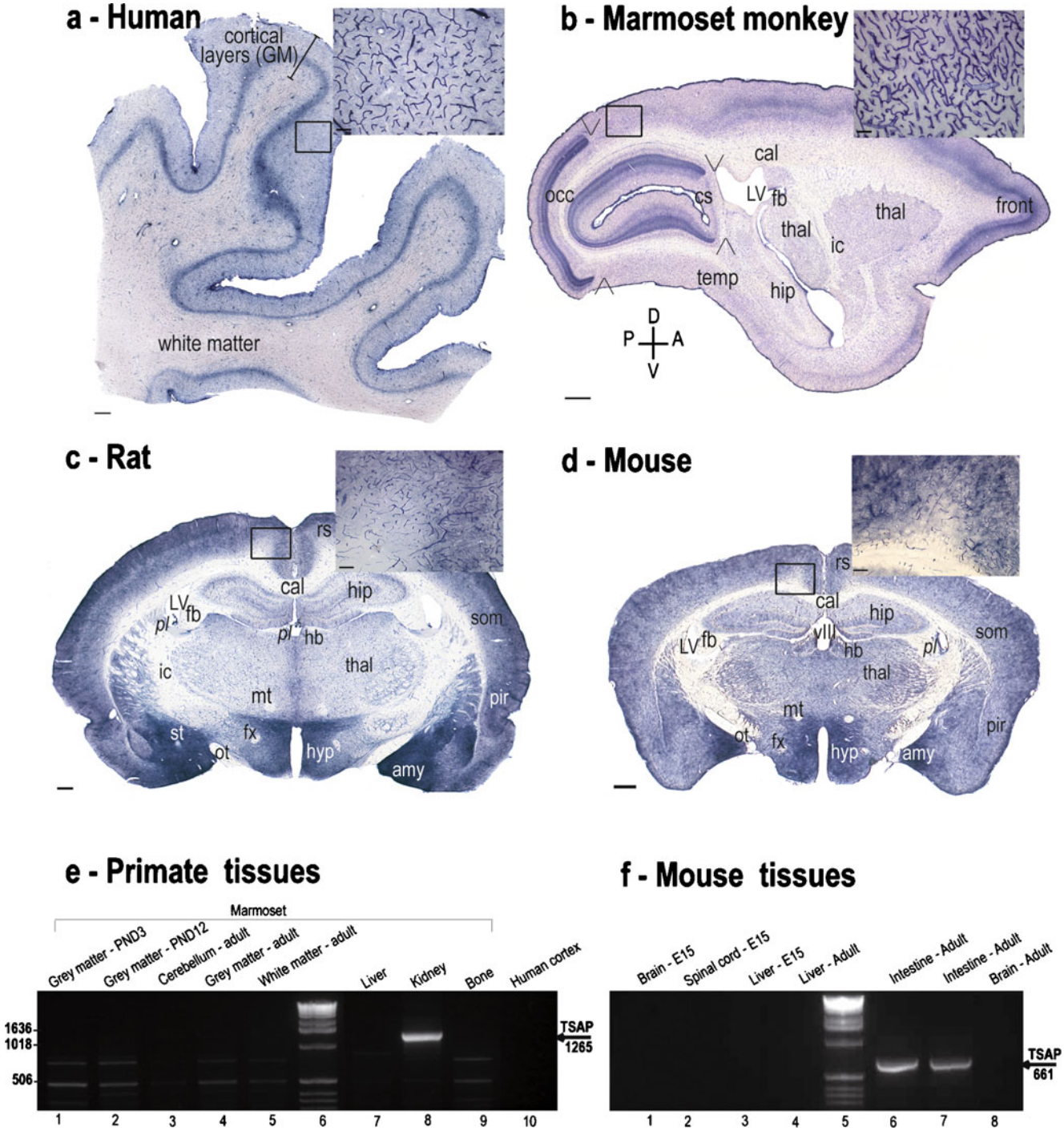


Fig. 2 AP activity in mammalian brains. AP activity in brain sections from primates (**a**, **b**) and rodents (**c**, **d**) was detected by histochemistry (*amy* amygdala, *cal* corpus callosum, *cs* calcarine sulcus, *fb* fimbria, *fx* fornix, *hb* habenula, *hip* hippocampus, *hyp* hypothalamus, *ic* internal capsule, *mt* mammillo-thalamic tract, *ot* optic tract, *pir* pirifrom cortex, *pl* choroid plexus, *rs* retro-spinal cortex, *som* somatosensory cortex, *st* commissural stria terminalis, *thal* thalamus, *GM* grey matter, *LV* lateral ventricle, *vIII* third ventricle). For primates, a section of human prefrontal cortex (**a**) and a para-sagittal section of an adult marmoset brain (**b**) are presented (*D* dorsal, *V* ventral, *P* posterior, *A* anterior). The various cortical regions correspond to the occipital (*occ*), temporal (*temp*) and frontal (*front*) cortex and the primary visual cortex (*arrowheads*). In the case of rodents, AP activity is shown in coronal sections of adult rat (**c**) and adult mouse (**d**) brains. *Boxed areas* in **a**–**d** Intra-cerebral vessels are AP-positive in all species, as illustrated at higher magnification in the *insets*. *Bars* 500 μm (**a**–**d**), 50 μm (*insets*). Expression of tissue-specific APs was explored by PCR in the nervous tissue in primates (**e**) and in rodents (**f**). They were not expressed in adult or young marmoset brains (**e**, *lanes 1-5*) and human cortex (**e**, *lane 10*) or in the nervous tissues of mouse embryo (**f**, *lanes 1*, *2*) and adult (**f**, *lane 8*). They were also excluded from tissues such as liver and bone, which express tissue non-specific AP (**e**, *lanes 7*, *9*, **f**, *lanes 3*, *4*). Positive controls are shown in **e** (*lane 8*), **f** (*lanes 6*, *7*); *lane 6* in **e**, *lane 5* in **f** 1-kb DNA ladder (Invitrogen)

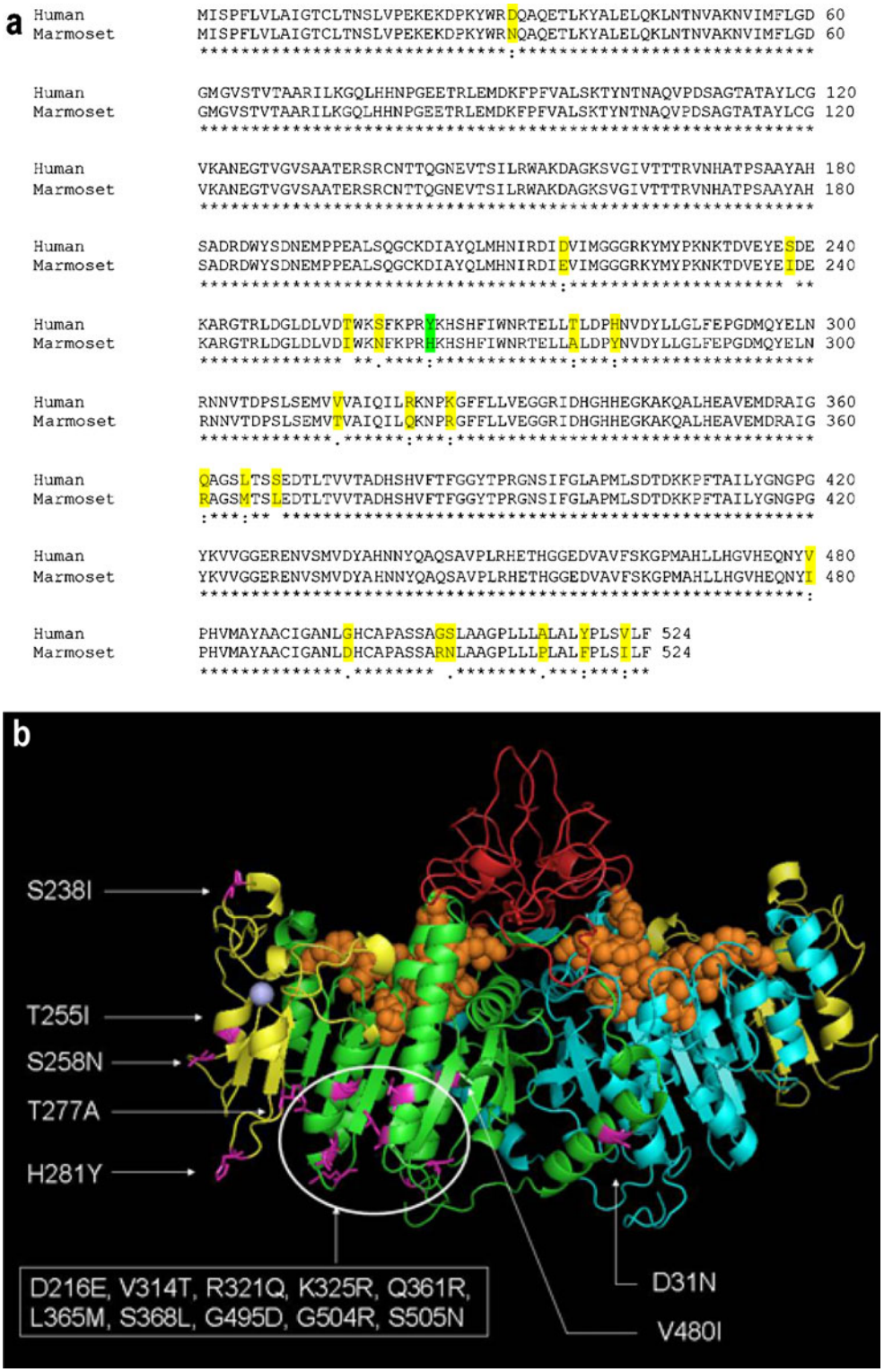


Fig. 3 Comparison of marmoset and human TNAP sequences. **a** Alignment of the marmoset TNAP (GenBank submission number GQ465850) with human TNAP. The 20-amino-acid differences between the two proteins are shown in *yellow*, except the change H>Y at position 263 (*green*), since it corresponds to a human polymorphism (Henthorn et al. 1992; *ALPL* gene mutation database, 2009, http://www.sesep.uvsq.fr/Database.html). **b** Positions of the amino acid residues differing between human and marmoset TNAP (indicated as human residue/position number/marmoset residue) are shown on the three-dimensional functional TNAP dimeric-model (Mornet et al. 2001). The two monomers are shown in *green* and *cyan blue*, respectively. The differing amino-acid residues (*magenta*) are found clustered in two specific regions: the calcium-binding site of the enzyme (in *yellow* with the calcium atom as a *pale blue sphere*) and the floor of the molecule (centred in *white ellipse*). The TNAP active site is shown as *orange spheres* and the crown domain as a *red line*

appear in the model of the mature protein (positions A514P, Y518F, V522I), (2) at the surface of the calcium-binding site (positions T255I, S238I, S258N, T277A, H281Y) and (3) at the surface of the floor of the molecule (positions D216E, V314T, R321Q, K325R, Q361R, L365M, S368L, G495D, G504R, S505N). The last two amino-acids (D31N and V480I) are not located in any functional domain.

Importantly, RACE analysis showed that exon 1 of the TNAP transcript expressed in marmoset brain was highly similar (94.4% nucleotide identity) to human exon 1A,

driven by the bone promoter. When making a comparison among the *ALPL* exon 1As predicted from the genome sequences of other primates (chimpanzee, orangutan and Rhesus macaque), the identity ranged from 91% to 94%. Extension of the aligned region to include the 2-kb ALPL bone promoter sequence and a double-check of the identity with the predicted homologous regions in the other primate species resulted in identities ranging from 86% to 99%, indicating the high degree of conservation of this region (Electronic Supplementary Material, Fig. S1).

This prompted us to investigate further whether TNAP transcripts expressed in cerebral tissues of different species were also of the "bone"-type. We used TNAP species-specific primers (Fig. 1) to amplify either the bone-type (exon 1A) or the liver-type (exon 1B) leader exon 1. In agreement with our primer extension data, the bone TNAP transcript (B/TNAP) was detected in marmoset brain in the cerebrum (Fig. 4b, lane 1), in white-matter-enriched (Fig. 4a, lane 4) and in grey-matter-enriched (Fig. 4a, lane 5) extracts and in the cerebellum (Fig. 4a, lane 3). This finding also applied to human cortex (Fig. 4a, lane 2). In rodents, the bone TNAP transcript was also detected in rat (Fig. 4b, lane 4) and mouse (Fig. 4b, lane 6) brain. Notably, the bone transcript was also detected in the liver of these species (Fig. 4b, lanes 2, 5, 7).

The liver TNAP transcript (L/TNAP) was found, as expected, in the liver of marmoset, rat and mouse (Fig. 4c, lanes 2, 5, 7, respectively). Notably, in the liver in which both liver and bone promoters were found to be used in all species studied, we observed differences in the relative proportions of bone and liver mRNA according to the animal species. For example, bone and liver mRNA levels were similar in monkey (Fig. 4b, c, lane 2), whereas bone mRNA prevailed in rat (Fig. 4b, c, lane 5) and liver mRNA in mouse liver (Fig. 4b, c, lane 7). However, L/TNAP was not detected in marmoset (Fig. 4c, lane 1), rat (Fig. 4c, lane 4) or human brain (data not shown). Strikingly, the liver-specific TNAP fragment was clearly detected in mouse brain (Fig. 4c, lane 6).

Our data thus demonstrated that the bone TNAP transcript was present in the adult brain of various mammalian species. They also pointed out the additional expression of the liver TNAP transcript only in mouse brain.

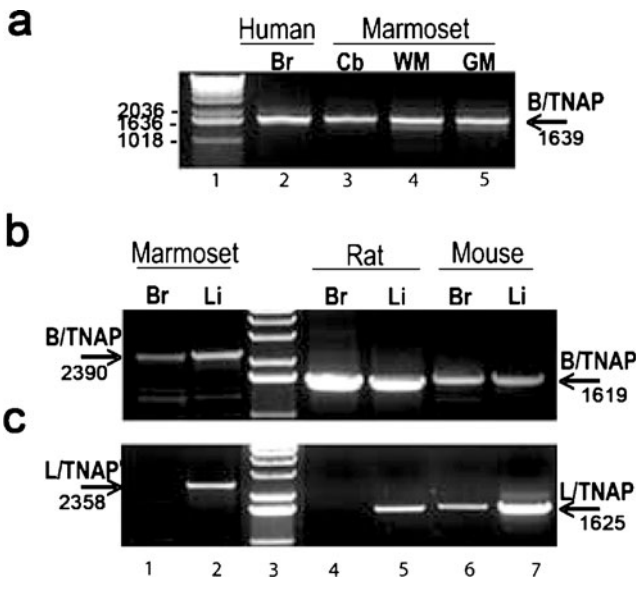


Fig. 4 PCR analyses of bone and liver TNAP isoforms expression in primates and rodents. Bone TNAP (*B/TNAP*) expression (**a**) in cerebral tissue of marmoset, such as cerebellum (*Cb*, *lane 3*), or in cerebral extracts enriched in white matter (*WM*, *lane 4*) or cortical grey matter (*GM*, *lane 5*) compared with human brain extracts (*lane 2*). Bone TNAP (*B/TNAP*) expression (**b**) and liver TNAP (*L/TNAP*) expression (**c**) was determined in brain (*Br*) and liver (*Li*) of marmoset (*lanes 1*, *2*), rat (*lanes 4*, *5*) and mouse (*lanes 6*, *7*). *Lane 1* in **a**, *lane 3* in **b**, **c** DNA ladder (1-kb DNA ladder, Invitrogen). *Arrows* indicate bone-specific (*B/TNAP*) and liver-specific (*L/TNAP*) fragments revealed by using species-specific primers of the two TNAP transcripts (cf. Fig. 1) and their corresponding sizes

Liver and bone TNAP transcripts are found at early developmental stages

We then investigated whether the differential expression of the bone and liver TNAP observed in rodents as compared with those of primates also applied during development. In monkey, the patterns of AP activity revealed in adulthood by histochemistry did not change (Fonta et al. 2004). However, the differential AP activity observed in brain sections revealed developmental changes occurring between neonate and adult monkeys. These studies at various postnatal ages, viz. one week apart for the first three postnatal months (Fonta et al. 2005), showed an extinction of AP activity in the white matter and an increase in the grey matter. Levamisole completely inhibited this activity in the white matter, in the grey matter and in endothelial cells at birth and in adults (Fonta et al. 2004, 2005). These results were further explored by a comparative analysis of TNAP expression between infant and adult ages. This analysis showed that, at both PND-3 and PND-12 stages, cerebral tissue expressed TNAP transcript of exon 1A (data not shown, Table 1). Similarly to the adult age, no tissue-specific AP was detected in neonates (Fig. 2e, lanes 1, 2).

We compared TNAP expression in rodent embryos and adults. We chose the E15 stage in which TNAP activity had previously been detected in both rat and mouse (Langer et al. 2007; Tam and Kwong 1987), and whose developmental time course did not greatly differ between these two species (1 day on average; Schneider and Norton 1979). At the E15 developmental stage in rat (Fig. 5b), as in mouse (Fig. 5a), histochemical analysis revealed particularly important AP

Table 1 TNAP transcript expression in various tissues and species (*B* bone TNAP, *L* liver TNAP, *0* no TNAP)

Tissue/cell type	Species	TNAP transcripts	Figures
Adult cerebral tissue	Human	B	2, 4
	Marmoset	B	2, 3, 4
	Rat	B	2, 4
	Mouse	B/L	2, 4, 5
Adult cerebellum	Marmoset	B	4
Adult white matter	Marmoset	B	4
Adult grey matter	Marmoset	B	4
Embryonic day 15 (E15) brain	Mouse	B/L	2, 5
E15 spinal cord	Mouse	B/L	2, 5
E15 neurons	Rat	B	5
E15 glial cell	Rat	B	5
Postnatal day 3 (PND 3) grey matter	Marmoset	B	Not shown
PND 12 grey matter	Marmoset	B	Not shown
Neuroepithelial precurseur 1C11	Mouse	0	6
Serotonergic neurons $1C11^{-5HT}$	Mouse	B/L	6
Noradrenergic neurons $1C11^{-NE}$	Mouse	B/L	6
Brain endothelial cells (hCMEC/D3)	Human	B	6
Brain endothelial cells (pMBMEC)	Mouse	B	6
Cultured pMBMEC	Mouse	0	6
Adult liver	Marmoset	L/B	4
	Rat	L/B	4
	Mouse	L/B	2, 4, 5
E15 liver	Mouse	B	2, 5

activity in brain and spinal cord. Moreover, it showed variations in the intensity of AP activity with, for example, more activity in the pons, ventral part of the spinal cord or adenohypophysis than in the thalamus, telencephalon, midbrain and hypothalamus (cf. strong AP activity in the adult hypothalamus, see Fig. 2c, d). AP activity was observed in fibres originating from the spinal cord or the olfactory mucosa, but no fibres were stained in adults (see Fig. 2). The choroid plexus also appeared to be AP-positive in both rat and mouse; however, vessels were AP-positive only in the rat embryo nervous tissue. In the other embryonic tissues, strong AP activity was observed in some bones (sphenoid bone, mandible), in cartilage (at the level of the vertebrae and the larynx in mouse), in lungs and in glands (thymus), whereas weak activity was observed in liver and muscles (heart, diaphragm).

Our PCR analyses fully corroborated these data by demonstrating that TNAP was expressed in E15 mouse brain and in spinal cord (Fig. 5c). Interestingly, the peculiarity described in adult mouse brain of a dual expression of bone and liver TNAP isoforms (Fig. 5c, lanes 5, 11, Fig. 4b, c, lane 6) was also observed in mouse embryonic brain (Fig. 5c, lanes 1, 7) and spinal cord (Fig. 5c, lanes 2, 8). In the embryo (Fig. 5c, lanes 1, 2 versus lanes 7, 8), as in the adult (Fig. 5c, lane 5 versus lane 11), the bone TNAP transcript appeared to be expressed at a higher level than the liver TNAP transcript in nervous tissue. No transcript of TSAP was detected in the embryonic rodent brain or spinal cord (Fig. 2f, lanes 1, 2), similar to results obtained in adult brain (Fig. 2f, lane 8). The liver TNAP transcript was not detected in embryonic mouse liver (Fig. 5c, lane 9), whereas in the adult, this transcript was found at a higher level than the bone transcript (Fig. 5c, lane 10 versus lane 4). This suggested that a drastic increase of the liver TNAP isoform expression occurred concomitantly with a decrease of the bone transcript in the liver at late embryonic stages or at a postnatal developmental stage.

In rat, the bone transcript, identified in total adult brain (Fig. 4b, lane 4) was also found in neuronal and glial cell preparations derived from E15 cortex (Fig. 5d). Importantly, no fragment corresponding to the liver transcript was amplified in the rat cerebral cells (data not shown).

Together, our results shed light on the fine regulation of TNAP expression depending on the animal species, the organ and the developmental stage.

"Liver" TNAP together with "bone" TNAP is expressed by mouse neuronal cells, whereas brain endothelial cells only express the bone transcript

The finding of both bone and liver TNAP transcripts in whole mouse brain extracts (Figs. 4b, c, 5c) led us to check

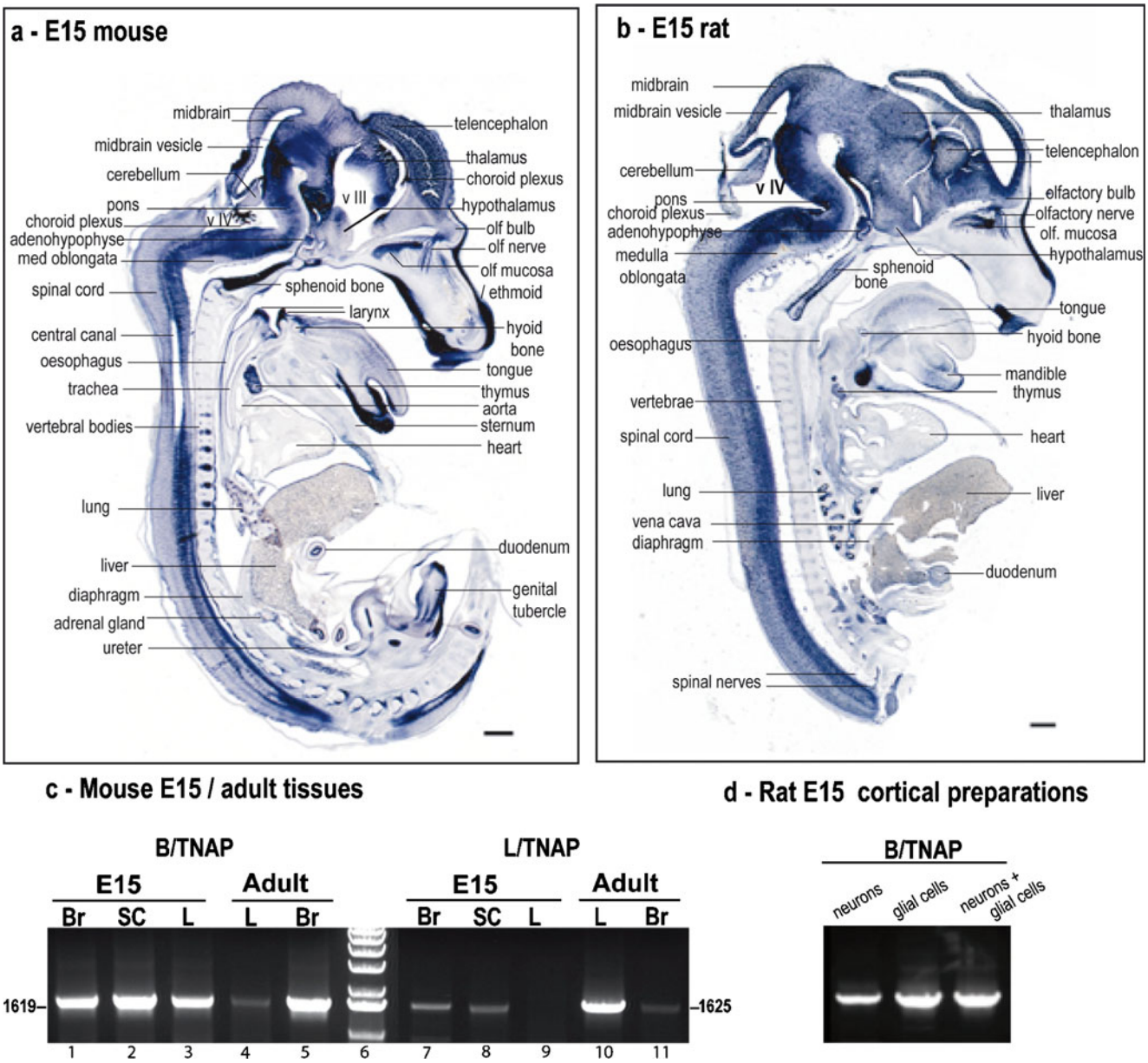


Fig. 5 Developmental expression of TNAP in rodents. Intense AP activity detected by histochemistry in rodent embryos, especially in the neuronal tissue in para-sagittal E15 mouse (**a**) and rat (**b**) embryo sections. *Bars* 500 μm. Analysis of TNAP transcripts (**c**) of the bone (*B/TNAP*, *lanes 1-5*) or liver (*L/TNAP*, *lanes 7-11*) transcript present in extracts from mouse liver (*L*) or nervous tissue, i.e. brain (*Br*) and spinal cord (*SC*), in E15 embryo or adult mice. *Lane 6* corresponds to the DNA ladder (1-kb DNA ladder, Invitrogen). The expected sizes of B/TNAP and L/TNAP amplified fragments in mouse (respectively, 1619 and 1625 bp) are indicated *left* and *right*. Analysis of TNAP transcript (**d**) expressed in primary neurons or glial cells culture obtained from E15 rat brain

whether the liver TNAP transcript was expressed by cells of neuronal and/or endothelial origin. We thus used the well-characterized 1C11 mouse neuronal differentiation model (Mouillet-Richard et al. 2000) in which TNAP has been recently identified (Ermonval et al. 2009). Consistent with the published results, TNAP transcripts were found in the fully differentiated serotonergic ($1C11^{5\text{-HT}}$) or noradrenergic ($1C11^{NE}$) neuronal cells and not in their precursors (Fig. 6a). However, the present data added new information by indicating that both the bone and the liver isoforms were found in $1C11^{5\text{-HT}}$ (Fig. 6, lanes 2, 5) and in $1C11^{NE}$ (Fig. 6, lanes 3, 6) bioaminergic neurons. These results supported the data obtained with whole mouse cerebral tissue (cf. Figs. 4b, c, 5c) in which both transcripts were detected.

We next addressed the question of whether mouse endothelial cells could also express both TNAP isoforms. Ultrastructural analyses had indeed shown that AP activity

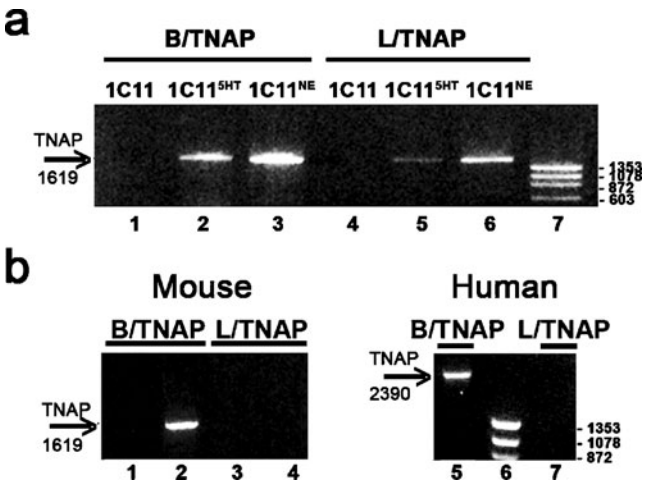


Fig. 6 TNAP expression in neuronal and brain endothelial cell lines. **a** PCR analysis of bone (*B/TNAP*) and liver (*L/TNAP*) TNAP expression in the 1C11 mouse neuroepithelial precursor cell line (*lanes 1*, *4*) and its $1C11^{5\text{-HT}}$ serotonergic (*lanes 2*, *5*) and $1C11^{NE}$ noradrenergic (*lanes 3*, *6*) neuronal progenies. **b** *Left* Gel with TNAP trancripts of the bone and liver types detected in pMBMEC mouse endothelial cell preparations freshly extracted from brain microvessels (*lanes 2*, *4*) or kept for a few days in culture (*lanes 1*, *3*). *Right* Gel showing TNAP expression in the hCMEC/D3 human endothelial cell line endowed with blood-brain-barrier properties (*lanes 5*, *7*). *Lane 7* in **a**, *lane 6* in **b** *Hae*III DNA restriction fragments of the Phi X bacteriophage. Markers sizes are indicated *right* (*arrows left* TNAP fragments and their expected sizes)

in vessel walls was localized at the extra-cellular surface of endothelial cells (Fonta et al. 2004). We only found the bone TNAP transcript in freshly isolated endothelial cells from mouse brain vessels (Lyck et al. 2009) (Fig. 6b, lane 2), whereas no fragment specific for the liver transcript was amplified (Fig. 6b, lane 4). Of note, TNAP was down-regulated when these endothelial cells were cultured for several days (Fig. 6b, lane 1). As in mouse, the previously characterized human endothelial cell line, hCMEC/D3 (Weksler et al. 2005), only expressed the bone TNAP transcript (Fig. 6b, lane 5).

Together, these data demonstrated that, in human and in various animal species, the bone promoter was preferentially used to drive the expression of TNAP in cells of neuronal and endothelial origin in the brain. However, the mouse was peculiar in that the liver TNAP promoter was also activated in the brain, but solely in neuronal cells.

Discussion

The present study unambiguously demonstrates that AP activity detected in brain tissues results from the expression of TNAP both in endothelial and neuronal cells. In addition, our data specify the TNAP promoters used in the cerebral tissue of rodents and primates. By taking advantage of our marmoset model, we show that no "neuronal specific" promoter for TNAP expression exists in the brain. However, whereas the bone-type TNAP is detected in the brain of all species tested, the liver TNAP mRNA has been specifically identified in mouse neuronal cells. Our results also suggest that TNAP transcription is regulated throughout development and neuronal differentiation.

In addition, our RACE mRNA analysis has allowed us to determine precisely the mRNA sequence of the marmoset TNAP expressed in the CNS; this sequence, as expected, closely resembles that of human. Interestingly, the amino acid residues that differ between these two species are clearly clustered into three regions: the surface of the calcium-binding site, the surface of the floor of the molecule and the hydrophobic GPI-anchoring signal of the molecule. The TNAP calcium site might represent a novel feature common to all mammalian AP and be essential for the enzyme activity and specificity (Brun-Heath et al. 2005; Millan 2006). Three of the changes in the calcium site involve the replacement of hydrophobic amino acids in the marmoset by polar amino acids in human, suggesting that ligands associated with the calcium site might differ between the two species. The floor of TNAP is supposed to contact the GPI anchor and the functional role of this domain is supported by evidence that this area harbours a great number of mutations responsible for severe hypophosphatasia (Mornet 2008). The same clusters of sequence variations are observed when human TNAP is compared with macaque TNAP (*Macaca mulatta*, Genbank accession number XM_001109717). This might reflect phylogenetic differences.

Although AP activity in the cerebral parenchyma was noted several decades ago, its brain patterns have only recently been precisely delineated in various mammals (Fonta et al. 2004, 2005; Langer et al. 2007, 2008). Our study indicates that AP patterns differ greatly in adult primates and rodents. For example, AP activity is found exclusively in one or a few cortical layers in several cerebral cortices but not in all cortical areas in primates (Fonta et al. 2004), whereas it is widely scattered, with diverse intensity levels, through the cortical depth and also in sub-cortical structures of the rodent brain (Langer et al. 2008). Such order-specific patterns raise the question with regard to specifying the cellular network context associated with AP activity in brain tissue. Our TNAP identification at the mRNA level correlates well with present and past structural analyses in adult brain: the TNAP mRNAs have been found in the grey matter and, surprisingly, in the white matter in which TNAP has not been detected by histochemistry. White matter is constituted by the myelinated axons connecting various brain structures. However, AP activity has been detected in this compartment by electron

microscopy, but this localization is limited to the nodes and paranodes of Ranvier in the white matter, i.e. axonal segments free of dense myelin (Fonta et al. 2005; Mori and Nagano 1985; Scherer 1996). Therefore, the apparent contradiction in the results might be explained by the different sensitivities of the employed techniques.

The analysis of TNAP expression in rodent and primate brain has allowed us to specify the identity of the ectophosphatase in neuronal and in endothelial cells. First, we have demonstrated that TNAP with exon 1A is expressed in various amount in neuronal cells. Moreover, a striking observation concerns TNAP expression in mouse neuronal cells. In addition to the bone TNAP transcript, mouse neurons also use the exon 1B promoter to drive TNAP expression. Importantly, this result increases our knowledge concerning TNAP isoform distribution in animal tissues and cell types. Whereas the bone TNAP is found in many different tissues, the present study has demonstrated that both transcripts are expressed in mouse neuronal cells. This result differs from the general idea that the two TNAP isoforms are expressed independently, except in the diaphragm in which the liver transcript has been reported contemporaneously with the bone transcript (Studer et al. 1991). Second, with respect to endothelial cells, AP activity detected in brain vessels has, until now, been attributed to TNAP on an indirect basis (Zoellner and Hunter 1989). We have shown here that brain endothelial cells, either of mouse (pMBMEC) or human (hCMEC/D3) origin, specifically express the TNAP isoform driven by the promoter specific for bone exon 1A. A role of TNAP in blood-brain-barrier (BBB) integrity endowed with transport systems or in a structural BBB element such as tight junctions is supported by data from the literature (Anstrom et al. 2007; Calhau et al. 2002; Risau et al. 1986). In addition, as observed for other BBB markers (Lyck et al. 2009), our data indicate the strong down-regulation of TNAP expression when pMBMEC mouse brain endothelial cells are maintained in culture for a few days, whereas in the human hCMEC/D3 endothelial cell line that retains its BBB characteristics in vitro (Weksler et al. 2005), we have detected bone TNAP transcripts under various culture conditions (data not shown).

Glial cells, because of their privileged relationship with brain vessels, are believed to play an important role in BBB-specific phenotypes by either increasing TNAP activity (through basic fibroblast growth factor) in brain endothelial cells (Sobue et al. 1999) or down-regulating TNAP expression (through transforming growth factor-beta) in brain micro-vessels (Nakazato et al. 1997). Moreover, an interleukin-6 (IL-6)-responsive element has been identified in the 5′-UTR of TNAP, reinforcing the idea of a role of IL-6 secretion by glial cells in BBB maintenance (Nakazato et al. 1997) and TNAP regulation (Takemoto et al. 1994). Our finding of a high level of TNAP transcripts in glial cells also corroborates the previous detection of AP activity at the surface of glia endfeet in newborn rat cortex and in glial cell membrane in adult rat cortex (Mayahara et al. 1967; Ovtscharoff 1973).

In addition to the cell and species specificity described here, TNAP regulation also occurs at a developmental level. This is exemplified by the induction of TNAP expression in 1C11-derived cells that have entered serotonergic or noradrenergic neuronal differentiation. Moreover, whereas neuronal TNAP is expressed by using the bone promoter, in all species studied at pre- and postnatal ages and in adulthood, our data indicate an age-dependent activation of the two TNAP promoters in mouse according to the organ. When comparing E15 embryo and adult mouse, the relative abundance of the bone and the liver TNAP mRNA seems to vary in an opposite way in neuronal and liver tissues. Finally, the strong AP enzymatic activity noted in rodent embryo sections in various structures of the brain, in the spinal cord and also in spinal nerves and olfactory cells, and partially reported in mice (Narisawa et al. 1994), has been confirmed by the expression of TNAP mRNA in E15 embryo brain and neurons. This agrees well with the relationship described for TNAP activity and neuronal progenitor cells (Langer et al. 2007).

Whereas much has still to be learned concerning the role of TNAP in the brain, evidence is increasing that it is involved in the CNS. The variation of TNAP expression and relative activity described here in diverse cellular models, tissues, organisms and developmental stages suggest complex regulatory mechanisms, possibly occurring at different levels. First, this might depend on cellular factors regulating TNAP expression. Only a few factors have been described that activate TNAP transcription. Retinoic acid (RA), which interestingly plays an essential role in pre- and postnatal development of the nervous tissue (Smith et al. 2001), has been shown to up-regulate bone TNAP, but not liver TNAP, in human and mouse (Escalante-Alcalde et al. 1996; San Miguel et al. 1999; Studer et al. 1991) through an RA-responsive element (RARE; Heath et al. 1992; Orimo and Shimada 2005; Scheibe et al. 1991). Vitamin D has also been shown to stimulate bone TNAP gene expression in osteoblast-like cells, possibly by binding to vitamin-D-regulating elements (VDRE; Johnson-Pais and Leach 1996; Kyeyune-Nyombi et al. 1991). Notably, vitamin D participates in neuronal differentiation and neuroprotection by interacting with specific receptors expressed in various brain areas (Taniura et al. 2006). In this context, our sequence analysis of the brain TNAP promoter of the marmoset and sequence alignments has revealed the presence of both RARE and VDRE at highly conserved positions in the bone TNAP promoter of various primates (Electronic Supplementary Material, Fig. S1). Concerning exon 1B, dibutyryl cAMP

(Studer et al. 1991) and, to a lesser extent, dexamethasone (Zernik et al. 1991) have been reported to increase liver TNAP transcription in vitro. An understanding of the way that one or other of the TNAP promoters is influenced by the cellular environment in a cell- and species-dependent way, as pointed out by our results, will be of interest.

Second, TNAP activity has also been shown to be modulated by post-translational modification, such as glycosylation (Halling Linder et al. 2009; Kiledjian and Kadesch 1991; Mueller et al. 2000). The impact of glycosylations on protein biosynthesis makes it unclear whether their effects on TNAP activity are only direct. However, the animal species and tissue specificity of added N-glycosylation structures are expected to lead to TNAP glycoprotein pools that might vary in different cell contexts and be associated with functional variation.

Finally, TNAP function presumably also relies on its substrate specificity and availability. The main enzymatic function of TNAP is as a phosphomonoesterase generating inorganic phosphate. In the brain, the natural TNAP substrate is pyridoxal phosphate (PLP), a form of vitamin B6 and a co-factor of GABA-synthesizing (Whyte et al. 1985) and sphingosine-synthesizing enzymes (Martin et al. 1991; Stephens and Dakshinamurti 1975). This correlates well with reports of epilepsy both in human cases of hypophosphatasia and in TNAP-deficient mice in which decreased levels of GABA (Waymire et al. 1995) and abnormal lumbar nerve roots, probably attributable to defects in myelination (Narisawa et al. 1997), have been recorded. PLP is also a cofactor of the aromatic amino acid decarboxylase (AADC) required for bioamine synthesis (serotonin, dopamine), and an involvement of TNAP in the metabolism of these neurotransmitters has been described (Ermonval et al. 2009). This enlarges the role of TNAP to other neuronal functions as supported by recent clinical and metabolic investigations revealing functional AADC deficiency in patients with hypophosphatasia and neonatal epileptic encephalopathy (Balasubramaniam et al. 2010). Of note, TNAP also has an ectonucleotidase activity that might participate in the control of nucleotide availability in the brain, such as the adenosine neuroprotective agent. This could also confer roles in developmental processes, such as cell growth, migration and differentiation and in neurotransmission (Neary and Zimmermann 2009; Zimmermann 2006). In addition, TNAP exhibits putative functional domains that might be involved in protein interactions, in particular with collagen (Millan 2006). Therefore, this ecto-phosphatase is suspected to act on matrix or membrane phosphoproteins. Indeed, recent data illustrate such functions of TNAP on neuronal extracellular matrix via its activity on phospholaminin (Ermonval et al. 2009) or through the dephosphorylation of extracellular tau (Diaz-Hernandez et al. 2010).

Our study, together with the diverse levels of TNAP regulation, indicate the importance of searching for the regulatory conditions and factors that enable TNAP to exhibit its different functions depending on its environment.

In conclusion, our results strongly support the hypothesis that the AP activity reported in rodent and primate brains results from TNAP gene transcription and that impairment in neurological functions and brain development, as observed in TNAP-deficient individuals, might arise from a direct effect of failure of TNAP. The present contribution should allow the design of effective tools to analyse TNAP expression by in situ hybridization in various species and to evaluate cellular factors involved in its complex regulation. This should help in characterizing the neuronal cells that express TNAP and the relationship between this ectophosphatase and neuronal functions.

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References

Anstrom JA, Brown WR, Moody DM, Thore CR, Challa VR, Block SM (2002) Temporal expression pattern of cerebrovascular endothelial cell alkaline phosphatase during human gestation. J Neuropathol Exp Neurol 61:76–84

Anstrom JA, Thore CR, Moody DM, Brown WR (2007) Immunolocalization of tight junction proteins in blood vessels in human germinal matrix and cortex. Histochem Cell Biol 127:205–213

Balasubramaniam S, Bowling F, Carpenter K, Earl J, Chaitow J, Pitt J, Mornet E, Sillence D, Ellaway C (2010) Perinatal hypophosphatasia presenting as neonatal epileptic encephalopathy with abnormal neurotransmitter metabolism secondary to reduced co-factor pyridoxal-5′-phosphate availability. J Inherit Metab Dis. Epub ahead of print

Bell MA, Ball MJ (1985) Laminar variation in the microvascular architecture of normal human visual cortex (area 17). Brain Res 335:139–143

Brun-Heath I, Taillandier A, Serre JL, Mornet E (2005) Characterization of 11 novel mutations in the tissue non-specific alkaline phosphatase gene responsible for hypophosphatasia and genotype-phenotype correlations. Mol Genet Metab 84:273–277

Calhau C, Martel F, Pinheiro-Silva S, Pinheiro H, Soares-da-Silva P, Hipolito-Reis C, Azevedo I (2002) Modulation of insulin transport in rat brain microvessel endothelial cells by an ecto-phosphatase activity. J Cell Biochem 84:389–400

Coisne C, Dehouck L, Faveeuw C, Delplace Y, Miller F, Landry C, Morissette C, Fenart L, Cecchelli R, Tremblay P, Dehouck B (2005) Mouse syngenic in vitro blood-brain barrier model: a new tool to examine inflammatory events in cerebral endothelium. Lab Invest 85:734–746

Diaz-Hernandez M, Gomez-Ramos A, Rubio A, Gomez-Villafuertes R, Naranjo J, Miras-Portugal M, Avila J (2010) Tissue non-

specific alkaline phosphatase promotes the neurotoxicity effect of extracellular tau. J Biol Chem 285:32539–32548
Ehrlich YH, Davis TB, Bock E, Kornecki E, Lenox RH (1986) Ecto-protein kinase activity on the external surface of neural cells. Nature 320:67–70
el-Moatassim C, Dornand J, Mani JC (1992) Extracellular ATP and cell signalling. Biochim Biophys Acta 1134:31–45
Ermonval M, Baudry A, Baychelier F, Pradines E, Pietri M, Oda K, Schneider B, Mouillet-Richard S, Launay JM, Kellermann O (2009) The cellular prion protein interacts with the tissue non-specific alkaline phosphatase in membrane microdomains of bioaminergic neuronal cells. PLoS ONE 4:e6497
Escalante-Alcalde D, Recillas-Targa F, Hernandez-Garcia D, Castro-Obregon S, Terao M, Garattini E, Covarrubias L (1996) Retinoic acid and methylation cis-regulatory elements control the mouse tissue non-specific alkaline phosphatase gene expression. Mech Dev 57:21–32
Fonta C, Imbert M (2002) Vascularization in the primate visual cortex during development. Cereb Cortex 12:199–211
Fonta C, Negyessy L, Renaud L, Barone P (2004) Areal and subcellular localization of the ubiquitous alkaline phosphatase in the primate cerebral cortex: evidence for a role in neurotransmission. Cereb Cortex 14:595–609
Fonta C, Negyessy L, Renaud L, Barone P (2005) Postnatal development of alkaline phosphatase activity correlates with the maturation of neurotransmission in the cerebral cortex. J Comp Neurol 486:179–196
Halling Linder C, Narisawa S, Millan JL, Magnusson P (2009) Glycosylation differences contribute to distinct catalytic properties among bone alkaline phosphatase isoforms. Bone 45:987–993
Heath JK, Suva LJ, Yoon K, Kiledjian M, Martin TJ, Rodan GA (1992) Retinoic acid stimulates transcriptional activity from the alkaline phosphatase promoter in the immortalized rat calvarial cell line, RCT-1. Mol Endocrinol 6:636–646
Henthorn PS, Raducha M, Fedde KN, Lafferty MA, Whyte MP (1992) Different missense mutations at the tissue-nonspecific alkaline phosphatase gene locus in autosomal recessively inherited forms of mild and severe hypophosphatasia. Proc Natl Acad Sci USA 89:9924–9928
Hessle L, Johnson KA, Anderson HC, Narisawa S, Sali A, Goding JW, Terkeltaub R, Millan JL (2002) Tissue-nonspecific alkaline phosphatase and plasma cell membrane glycoprotein-1 are central antagonistic regulators of bone mineralization. Proc Natl Acad Sci USA 99:9445–9449
Johnson-Pais TL, Leach RJ (1996) 1,25-Dihydroxyvitamin D3 and transforming growth factor-beta act synergistically to override extinction of liver/bone/kidney alkaline phosphatase in osteosarcoma hybrid cells. Exp Cell Res 226:67–74
Kiledjian M, Kadesch T (1991) Post-transcriptional regulation of the human liver/bone/kidney alkaline phosphatase gene. J Biol Chem 266:4207–4213
Kruse K, Hanefeld F, Kohlschutter A, Rosskamp R, Gross-Selbeck G (1988) Hyperphosphatasia with mental retardation. J Pediatr 112:436–439
Kyeyune-Nyombi E, Lau KH, Baylink DJ, Strong DD (1991) 1,25-Dihydroxyvitamin D3 stimulates both alkaline phosphatase gene transcription and mRNA stability in human bone cells. Arch Biochem Biophys 291:316–325
Langer D, Ikehara Y, Takebayashi H, Hawkes R, Zimmermann H (2007) The ectonucleotidases alkaline phosphatase and nucleoside triphosphate diphosphohydrolase 2 are associated with subsets of progenitor cell populations in the mouse embryonic, postnatal and adult neurogenic zones. Neuroscience 150:863–879
Langer D, Hammer K, Koszalka P, Schrader J, Robson S, Zimmermann H (2008) Distribution of ectonucleotidases in the rodent brain revisited. Cell Tissue Res 334:199–217
Lyck R, Ruderisch N, Moll AG, Steiner O, Cohen CD, Engelhardt B, Makrides V, Verrey F (2009) Culture-induced changes in blood-brain barrier transcriptome: implications for amino-acid transporters in vivo. J Cereb Blood Flow Metab 29:1491–1502
Mabry CC, Bautista A, Kirk RF, Dubilier LD, Braunstein H, Koepke JA (1970) Familial hyperphosphatase with mental retardation, seizures, and neurologic deficits. J Pediatr 77:74–85
Martin DL, Martin SB, Wu SJ, Espina N (1991) Regulatory properties of brain glutamate decarboxylase (GAD): the apoenzyme of GAD is present principally as the smaller of two molecular forms of GAD in brain. J Neurosci 11:2725–2731
Matsuura S, Kishi F, Kajii T (1990) Characterization of a 5′-flanking region of the human liver/bone/kidney alkaline phosphatase gene: two kinds of mRNA from a single gene. Biochem Biophys Res Commun 168:993–1000
Mayahara H, Hirano H, Saito T, Ogawa K (1967) The new lead citrate method for the ultracytochemical demonstration of activity of non-specific alkaline phosphatase (orthophosphoric monoester phosphohydrolase). Histochemie 11:88–96
Millan JL (2006) Mammalian alkaline phosphatases. From biology to applications in medicine and biotechnology. Wiley-VCH, Weinheim
Moody DM, Thore CR, Anstrom JA, Challa VR, Langefeld CD, Brown WR (2004) Quantification of afferent vessels shows reduced brain vascular density in subjects with leukoaraiosis. Radiology 233:883–890
Mori S, Nagano M (1985) Electron microscopic cytochemistry of alkaline phosphatase in neurons of rats. Arch Histol Jpn 48:389–397
Mornet E (2008) Hypophosphatasia. Best Pract Res Clin Rheumatol 22:113–127
Mornet E, Stura E, Lia-Baldini AS, Stigbrand T, Menez A, Le Du MH (2001) Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization. J Biol Chem 276:31171–31178
Mouillet-Richard S, Mutel V, Loric S, Tournois C, Launay JM, Kellermann O (2000) Regulation by neurotransmitter receptors of serotonergic or catecholaminergic neuronal cell differentiation. J Biol Chem 275:9186–9192
Mueller WH, Kleefeld D, Khattab B, Meissner JD, Scheibe RJ (2000) Effects of retinoic acid on N-glycosylation and mRNA stability of the liver/bone/kidney alkaline phosphatase in neuronal cells. J Cell Physiol 182:50–61
Nakazato H, Deguchi M, Fujimoto M, Fukushima H (1997) Alkaline phosphatase expression in cultured endothelial cells of aorta and brain microvessels: induction by interleukin-6-type cytokines and suppression by transforming growth factor betas. Life Sci 61:2065–2072
Narisawa S, Hasegawa H, Watanabe K, Millan JL (1994) Stage-specific expression of alkaline phosphatase during neural development in the mouse. Dev Dyn 201:227–235
Narisawa S, Frohlander N, Millan JL (1997) Inactivation of two mouse alkaline phosphatase genes and establishment of a model of infantile hypophosphatasia. Dev Dyn 208:432–446
Neary JT, Zimmermann H (2009) Trophic functions of nucleotides in the central nervous system. Trends Neurosci 32:189–198
Negyessy L, Xiao J, Kantor O, Kovacs GG, Palkovits M, Doczi TP, Renaud L, Baksa G, Glasz T, Ashaber M, Barone P, Fonta C (2010) Layer-specific activity of tissue non-specific alkaline phosphatase in the human neocortex. Neuroscience. Epub ahead of print
Nouwen EJ, De Broe ME (1994) Human intestinal versus tissue-nonspecific alkaline phosphatase as complementary urinary markers for the proximal tubule. Kidney Int Suppl 47:S43–S51
Orimo H, Shimada T (2005) Regulation of the human tissue-nonspecific alkaline phosphatase gene expression by all-trans-retinoic acid in SaOS-2 osteosarcoma cell line. Bone 36:866–876

Ovtscharoff W (1973) Ultracytochemical localisation of the alkaline phosphatase in the cerebral cortex of newborn rats. Histochemie 37:93–95

Plesner L (1995) Ecto-ATPases: identities and functions. Int Rev Cytol 158:141–214

Rathbun JC (1948) Hypophosphatasia; a new developmental anomaly. Am J Dis Child 75:822–831

Risau W, Hallmann R, Albrecht U, Henke-Fahle S (1986) Brain induces the expression of an early cell surface marker for blood-brain barrier-specific endothelium. EMBO J 5:3179–3183

Robson SC, Sevigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430

San Miguel SM, Goseki-Sone M, Sugiyama E, Watanabe H, Yanagishita M, Ishikawa I (1999) Tissue-non-specific alkaline phosphatase mRNA expression and alkaline phosphatase activity following application of retinoic acid in cultured human dental pulp cells. Arch Oral Biol 44:861–869

Scheibe RJ, Ginty DD, Wagner JA (1991) Retinoic acid stimulates the differentiation of PC12 cells that are deficient in cAMP-dependent protein kinase. J Cell Biol 113:1173–1182

Scherer SS (1996) Molecular specializations at nodes and paranodes in peripheral nerve. Microsc Res Tech 34:452–461

Schmidt F, Champy P, Seon-Meniel B, Franck X, Raisman-Vozari R, Figadere B (2009) Chemicals possessing a neurotrophin-like activity on dopaminergic neurons in primary culture. PLoS ONE 4:e6215

Schneider BF, Norton S (1979) Equivalent ages in rat, mouse and chick embryos. Teratology 19:273–278

Smith D, Wagner E, Koul O, McCaffery P, Drager UC (2001) Retinoic acid synthesis for the developing telencephalon. Cereb Cortex 11:894–905

Sobue K, Yamamoto N, Yoneda K, Hodgson ME, Yamashiro K, Tsuruoka N, Tsuda T, Katsuya H, Miura Y, Asai K, Kato T (1999) Induction of blood-brain barrier properties in immortalized bovine brain endothelial cells by astrocytic factors. Neurosci Res 35:155–164

Stephens MC, Dakshinamurti K (1975) Brain lipids in pyridoxine-deficient young rats. Neurobiology 5:262–269

Studer M, Terao M, Gianni M, Garattini E (1991) Characterization of a second promoter for the mouse liver/bone/kidney-type alkaline phosphatase gene: cell and tissue specific expression. Biochem Biophys Res Commun 179:1352–1360

Takemoto H, Kaneda K, Hosokawa M, Ide M, Fukushima H (1994) Conditioned media of glial cell lines induce alkaline phosphatase activity in cultured artery endothelial cells. Identification of interleukin-6 as an induction factor. FEBS Lett 350:99–103

Tam PP, Kwong WH (1987) A study on the pattern of alkaline phosphatase activity correlated with observations on silver-impregnated structures in the developing mouse brain. J Anat 150:169–180

Taniura H, Ito M, Sanada N, Kuramoto N, Ohno Y, Nakamichi N, Yoneda Y (2006) Chronic vitamin D3 treatment protects against neurotoxicity by glutamate in association with upregulation of vitamin D receptor mRNA expression in cultured rat cortical neurons. J Neurosci Res 83:1179–1189

Thompson MD, Nezarati MM, Gillessen-Kaesbach G, Meinecke P, Mendoza R, Mornet E, Brun-Heath I, Squarcioni CP, Legeai-Mallet L, Munnich A, Cole DE (2010) Hyperphosphatasia with seizures, neurologic deficit, and characteristic facial features: five new patients with Mabry syndrome. Am J Med Genet A 152A:1661–1669

Toh Y, Yamamoto M, Endo H, Misumi Y, Ikehara Y (1989) Isolation and characterization of a rat liver alkaline phosphatase gene. A single gene with two promoters. Eur J Biochem 182:231–237

Van Belle H (1976) Alkaline phosphatase. I. Kinetics and inhibition by levamisole of purified isoenzymes from humans. Clin Chem 22:972–976

Waymire KG, Mahuren JD, Jaje JM, Guilarte TR, Coburn SP, MacGregor GR (1995) Mice lacking tissue non-specific alkaline phosphatase die from seizures due to defective metabolism of vitamin B-6. Nat Genet 11:45–51

Weiss MJ, Ray K, Henthorn PS, Lamb B, Kadesch T, Harris H (1988) Structure of the human liver/bone/kidney alkaline phosphatase gene. J Biol Chem 263:12002–12010

Weksler BB, Subileau EA, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male DK, Roux F, Greenwood J, Romero IA, Couraud PO (2005) Blood-brain barrier-specific properties of a human adult brain endothelial cell line. FASEB J 19:1872–1874

Wennberg C, Hessle L, Lundberg P, Mauro S, Narisawa S, Lerner UH, Millan JL (2000) Functional characterization of osteoblasts and osteoclasts from alkaline phosphatase knockout mice. J Bone Miner Res 15:1879–1888

Whyte MP (1994) Hypophosphatasia and the role of alkaline phosphatase in skeletal mineralization. Endocr Rev 15:439–461

Whyte MP, Mahuren JD, Vrabel LA, Coburn SP (1985) Markedly increased circulating pyridoxal-5′-phosphate levels in hypophosphatasia. Alkaline phosphatase acts in vitamin B6 metabolism. J Clin Invest 76:752–756

Yusa N, Watanabe K, Yoshida S, Shirafuji N, Shimomura S, Tani K, Asano S, Sato N (2000) Transcription factor Sp3 activates the liver/bone/kidney-type alkaline phosphatase promoter in hematopoietic cells. J Leukoc Biol 68:772–777

Zernik J, Kream B, Twarog K (1991) Tissue-specific and dexamethasone-inducible expression of alkaline phosphatase from alternative promoters of the rat bone/liver/kidney/placenta gene. Biochem Biophys Res Commun 176:1149–1156

Zimmermann H (2006) Nucleotide signaling in nervous system development. Pflugers Arch 452:573–588

Zoellner HF, Hunter N (1989) Histochemical identification of the vascular endothelial isoenzyme of alkaline phosphatase. J Histochem Cytochem 37:1893–1898