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Overexpression of tissue-nonspecific alkaline phosphatase increases the expression of neurogenic differentiation markers in the human SH-SY5Y neuroblastoma cell line

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ABSTRACT

Patients suffering from the rare hereditary disease hypophosphatasia (HPP), which is based on mutations in the *ALPL* gene, tend to develop central nervous system (CNS) related issues like epileptic seizures and neuropsychiatric illnesses such as anxiety and depression, in addition to well-known problems with the mineralization of bones and teeth. Analyses of the molecular role of tissue-nonspecific alkaline phosphatase (TNAP) in transgenic SH-SY5Y^{TNAPhigh} neuroblastoma cells compared to SH-SY5Y^{TNAPlow} cells indicate that the enzyme influences the expression levels of neuronal marker genes like RNA-binding protein, fox-1 homolog 3 (NEUN) and enolase 2, gamma neuronal (NSE) as well as microtubule-binding proteins like microtubule-associated protein 2 (MAP2) and microtubule-associated protein tau (TAU) during neurogenic differentiation. Fluorescence staining of SH-SY5Y^{TNAPhigh} cells reveals TNAP localization throughout the whole length of the developed projection network and even synapsin I co-localization with strong TNAP signals at some spots at least at the early time points of differentiation. Additional immunocytochemical staining shows higher MAP2 expression in SH-SY5Y^{TNAPhigh} cells and further a distinct up-regulation of tau and MAP2 in the course of neurogenic differentiation. Interestingly, transgenic SH-SY5Y^{TNAPhigh} cells are able to develop longer cellular processes compared to control cells after stimulation with *all-trans* retinoic acid (RA). Current therapies for HPP prioritize improvement of the bone phenotype. Unraveling the molecular role of TNAP in extraosseous tissues, like in the CNS, will help to improve treatment strategies for HPP patients. Taking this rare disease as a model may also help to dissect TNAP's role in neurodegenerative diseases and even improve future treatment of common pathologies.

Introduction

Hypophosphatasia is a rare multisystemic disease with enormous variations concerning age of clinical manifestation as well as variability and severity of symptoms. Molecular bases of HPP are various mutations in the *ALPL* gene (1p36.12, NCBI Gene-ID:249) which is coding for the ectoenzyme tissue-nonspecific alkaline phosphatase [1]. The predominant phenotype of this hereditary disease in its severe early onset manifestation is characterized by impaired bone mineralization and by disorganized growth plates with consecutive malformations [1]. Moreover, lung hypoplasia and functional impairment of the CNS , including epileptic seizures, may contribute to early death within weeks after birth [2]. Less severe forms of the disease with infantile, adolescent or even adult onset present with delayed bone mineralization, muscular problems like dynopenia, nephrocalcinosis, poor renal function or problems with the gastrointestinal tract [1]. Focusing on CNS-related issues, above all adult patients complain about sleep disturbances and neuropsychiatric

Abbreviations: 36B4, ribosomal protein, large, P0; AADC, aromatic L-amino acid decarboxylase; AD, Alzheimer's disease; ALPL/TNAP/Akp2, tissue-nonspecific alkaline phosphatase; AT, annealing temperature; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; BSA, bovine serum albumin; CNS, central nervous system; CNTNAP2, contactin associated protein-like 2; DCX, doublecortin; EEF1A, eukaryotic translation elongation factor 1 alpha 1; FBS, fetal bovine serum; GABA, gamma-aminobutyric acid; GAD, glutamate decarboxylase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPP, hypophosphatasia; MAP2, microtubule-associated protein 2; NEUN, RNA-binding protein, fox-1 homolog 3; NFASC, neurofascin; NPY, neuropeptide Y; NRP1, neuropilin 1; NRXN1, neurexin 1; NSE, enolase 2, gamma neuronal; P2X7, purinergic receptor P2X, ligand gated ion channel, 7; PBS, phosphate buffered saline; PGCs, primordial germ cells; PHOSPHO1, phosphatase, orphan 1; PI, protease inhibitor; PL, pyridoxal; PLP, pyridoxal 5′ phosphate; PRKCA, protein kinase c, alpha; RA, *all-trans* retinoic acid; RARβ, retinoic acid receptor β; RLU, relative light unit; ROBO2, roundabout, axon guidance receptor, homolog 2; SEM, standard error of mean; SEMA3A, semaphorin 3A; SVZ, subventricular zone; TAU, microtubule-associated protein tau.

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illnesses like increased nervousness, anxiety or depression (unpublished data of HPP patients' organization and our unpublished results). Such CNS-related symptoms in HPP underline the importance of TNAP enzyme in the neuronal system beyond its central and meanwhile quite well-understood role in bone metabolism.

Alkaline phosphatase is among others responsible for dephosphorylation of small molecule and polypeptide mineralization inhibitors such as inorganic pyrophosphate [3] and osteopontin [4] which finally balances and modulates the crystallization of hydroxyapatite [5]. As previously published by Narisawa et al. skeletal phenotypes in $Alpl^{-/-}$ mice (NCBI Gene-ID: 11647) can partly be attributed to accumulation of phosphorylated osteopontin [4]. Another key player in this system is the phosphatase PHOSPHO1 which supports the initiation of hydroxyapatite deposition in matrix vesicles whereas TNAP enables propagation of the newly formed crystals outside the vesicles [6,7]. As a consequence, combined ablation of PHOSPHO1 and TNAP completely prevents skeletal mineralization [7]. In addition, adenosine triphosphate (ATP), diphosphate (ADP) and monophosphate (AMP) are further substrates for TNAP [8,9] thus contributing to changes in the microenvironmental concentrations of purine-derived compounds that orchestrate purinergic signaling via a series of receptors and ligand-modulated channels as well as adenosine receptors. Purinergic signaling is relevant in several tissues including bone and the central nervous system [10].

No brain-specific transcripts of TNAP have been found so far as all analyzed neuronal and endothelial cells in humans, marmosets and rats solely express the bone specific *ALPL* transcript, whereas mouse neurons also express liver transcripts [11]. Moreover, expression of tissue-nonspecific alkaline phosphatase changes in accordance with the developmental stage of the brain [11]. In the human neocortex TNAP expression can be found in layer 5 throughout the frontal-, temporal- and occipital lobes [12]. Further, alkaline phosphatase activity is localized in the neuropile alongside the thalamo-cortical innervations in layer 4 of primary visual, auditory and somatosensory cortices and furthermore varies depending on sensory experience. Subcellular localization of TNAP enzyme is characterized by accumulation at synaptic junctions during maturation, its distribution matches GAD_{65} localization at presynaptic terminals and apart from that alkaline phosphatase is distributed alongside the axon solely in myelin-free parts [13,14]. Consistent with those results Hanics et al. published that TNAP knockout mice ($Akp2^{-/-}$) display severe dysfunctions as far as central processes like myelination and maturation of synapses are concerned [15]. Moreover, TNAP enzyme might influence migration of primordial germ cells (PGCs) and neurons due to its ability of interaction with extracellular matrix proteins like collagen and further regulates proliferation and differentiation of neural stem cells into neurons or oligodendrocytes [16,17]. Experiments with murine neurons isolated from hippocampus samples revealed a connection between TNAP and axonal growth. TNAP staining shows prominent signals in somatic and axonal regions. The strongest signals are located at axon terminals and branching points, whereas only weak signals could be detected in dendrites [18]. TNAP inhibition impairs axonal growth significantly, probably due to elevated ATP levels that activate P2X7 receptors at the growth cone, which prevents axonal growth. In contrast, no effects of TNAP inhibition could be seen on dendritic outgrowth [18]. Moreover, TNAP might influence neurogenesis during mouse brain development and expression was found in the subventricular zone in adult mouse brains, which is one of the few regions where adult neurogenesis takes place [19]. Above all, TNAP is crucial for vitamin B_6 metabolism as only the transport form pyridoxal (PL) is able to cross the blood–brain barrier and functional TNAP is indispensable for dephosphorylation of pyridoxal-5′-phosphate (PLP). PL is activated through rephosphorylation after passing the blood–brain-barrier and serves as a cofactor for enzymes playing a central role in neurotransmitter metabolism, like aromatic L-amino acid decarboxylase (AADC) and glutamate decarboxylase (GAD) which regulate synthesis of serotonin, dopamine, (nor-)/adrenalin and GABA [20–22]. A recent publication describes quite conserved TNAP localization in the retina across many species and therefore suggests a potential role for retinal neurotransmission [23].

In order to reveal further details about the molecular function of TNAP in neuronal systems we established a neuroblastoma cell line which stably over-expresses TNAP enzyme (SH-SY5Y^{TNAPhigh}) and compared its gene expression patterns and morphology with and without treatment with neurogenic differentiation media to parent cells (SH-SY5Y^{TNAPlow}). Using this human *in vitro* model turned out to be a very promising tool in order to confirm and extend knowledge on the role of alkaline phosphatase concerning neurogenic differentiation and maturation. Literature gives information about localization of TNAP in mouse brains [17,24] and about the enzyme's role during axonal growth in murine hippocampal neurons [18] as well as during differentiation of murine neural stem cells [16]. Information about the distribution in the human neocortex was gained from histological analysis [12,13]. Using our in vitro model, we are able to further dissect the role of TNAP on consecutive events during differentiation and maturation in neuronal cell lines of human origin.

We can demonstrate here that TNAP overexpression profoundly influences outgrowth of cellular processes and expression of neuronal differentiation markers during neuronal cell differentiation, indicating that TNAP is capable of modulating intercellular communication in the CNS. Interestingly only certain cells in layer 4 and layer 5 of the human neocortex are TNAP positive whereas surrounding cells are not [12,13]. Our established *in vitro* model enables us to analyze possible roles of those cells in human context and provides additional knowledge to previously published literature data gained in mouse models.

Materials and methods

Cell culture

Neuroblastoma cell line SH-SY5Y (ATCC:CRL-2266) was kindly provided by Prof. Lesch (Department of Psychiatry, Psychosomatics and Psychotherapy, University of Wuerzburg, Germany) and cultured in DMEM Ham's F12 media (Life Technologies GmbH, Darmstadt, Germany) supplemented with 10% heat inactivated FBS (Biochrom AG, Berlin, Germany), 100 nM sodium selenite and 50 μg/ml gentamicin (both Sigma-Aldrich, Munich, Germany). Cells grew constantly at 37 °C and 5% CO_2 and were negatively tested for mycoplasma contamination by DAPI staining.

Plasmids and transfection

SH-SY5Y^{TNAPlow} cells were transfected using the reagent Lipofectamin 2000 according to manufacturer's instructions (Invitrogen GmbH, Darmstadt, Germany) with a SSP1 (New England Biolabs GmbH, Frankfurt/Main, Germany) linearized pcDNA3.1-vector (Invitrogen GmbH, Darmstadt, Germany) containing the coding region of the human *ALPL* gene in order to establish the cell line SH-SY5Y^{TNAPhigh}. The overexpression construct was previously described in literature [25]. A second cell line, SH-SY5Y$^{pcDNA3.1}$, containing the empty vector, was established equivalently for control purposes. Both stable cell lines were cultured in media containing 300 μg/ml G418 (BD Biosciences Clontech, Heidelberg, Germany) for selection of cells containing the respective constructs.

Isolation of RNA

Total RNA was isolated with the Nucleo Spin RNA II Kit (Macherey-Nagel GmbH and Co. KG, Düren, Germany) according to manufacturer's instructions.