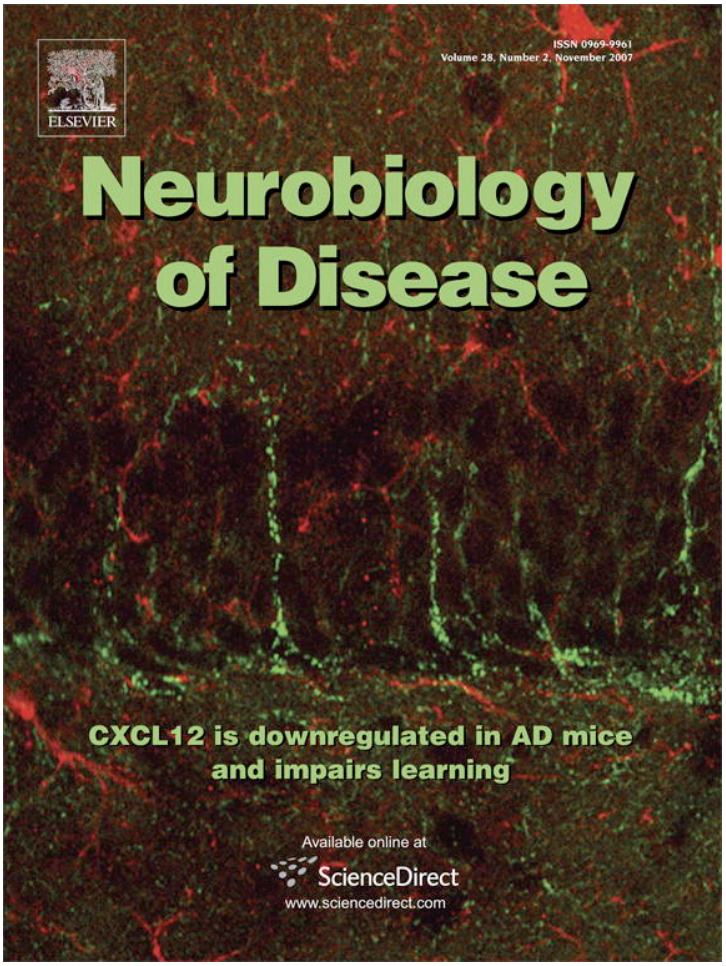

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Distribution and cellular localization of adrenoleukodystrophy protein in human tissues: Implications for X-linked adrenoleukodystrophy

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Defects of adrenoleukodystrophy protein (ALDP) lead to X-linked adrenoleukodystrophy (X-ALD), a disorder mainly affecting the nervous system white matter and the adrenal cortex. In the present study, we examine the expression of ALDP in various human tissues and cell lines by multiple-tissue RNA expression array analysis, Western blot analysis, and immunohistochemistry. ALDP-encoding mRNA is most abundant in tissues with high energy requirements such as heart, muscle, liver, and the renal and endocrine systems. ALDP selectively occurs in specific cell types of brain (hypothalamus and basal nucleus of Meynert), kidney (distal tubules), skin (eccrine gland, hair follicles, and fibroblasts), colon (ganglion cells and epithelium), adrenal gland (zona reticularis and fasciculata), and testis (Sertoli and Leydig cells). In pituitary gland, ALDP is confined to adrenocorticotropin-producing cells and is significantly reduced in individuals receiving long term cortisol treatment. This might indicate a functional link between ALDP and proopiomelanocortin-derived peptide hormones.

Keywords: Adrenoleukodystrophy protein (ALDP); X-ALD; Pituitary gland; Brain; Cortisol; Proopiomelanocortin (POMC)

Introduction

Adrenoleukodystrophy protein (ALDP) is a peroxisomal transmembrane protein, belonging to the family of ATP-binding cassette (ABC) transporters. The ALDP-encoding gene (*ABCD1*) was mapped to Xq28 and occupies approximately 26 kb of genomic DNA with 10 exons encoding 745 amino acids (Migeon et al., 1981; Mosser et al., 1993; Sarde et al., 1994).

Defects of ALDP due to mutations in *ABCD1* lead to X-linked adrenoleukodystrophy (X-ALD), a disease encompassing several remarkably different phenotypes such as cerebral ALD (cALD), in particular childhood cerebral ALD (ccALD), adrenomyeloneuropathy (AMN), as well as adrenal insufficiency (Berger and Gartner, 2006; Moser et al., 1991). The biochemical hallmark of X-ALD is an accumulation of very long chain fatty acids (VLCFAs), predominantly C26:0, in tissues and blood (Moser et al., 1980, 1981; Rasmussen et al., 1994). VLCFAs are oxidized by a set of β-oxidation enzymes, located in the peroxisomal matrix (Singh et al., 1984). It was first assumed that the defect in X-ALD involves the activation of fatty acids to acyl-CoA by the enzyme very long chain acyl-CoA synthetase (VLCS) (Hashmi et al., 1986; Lazo et al., 1988), later considerations included the possibilities that ALDP transports VLCFA, the VLCS enzyme, or a required cofactor across the peroxisomal membrane and thereby influences the peroxisomal β-oxidation. However, recent investigations could not find an alteration of peroxisomal β-oxidation in tissues of ALDP-deficient mice (McGuinness et al., 2003; Oezen et al., 2005) which argues against a direct participation of ALDP in the degradation of VLCFA (Berger and Gartner, 2006).

To disclose the physiological function of ALDP, data on the specific cellular distribution in normal human tissues might be helpful. Until now human *ABCD1* expression has been included in a broad range of study describing the expression pattern of all ABC transporters across several tissues (Langmann et al., 2003). On the protein level, expression of ALDP has been investigated in different mouse tissues and cell lines, showing a predominance in adrenal gland, kidney, lung and muscle (Troffer-Charlier et al.,

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1998). However, apart from one immunohistochemical description of ALDP expression in brain (Fouquet et al., 1997), histological examination of ALDP in human tissues is lacking. Therefore, we systematically investigate here a wide range of human tissues and cell lines using multiple-tissue array blot, Western blot analysis, immunohistochemistry, and double/triple immunofluorescence to demonstrate the normal expression pattern of *ABCD1* mRNA and protein.

Material and methods

Tissues

Formalin-fixed, paraffin-embedded and fresh frozen archival human autopsy and biopsy material from 36 individuals is collected at the Institute of Neurology and Department of Clinical Pathology, Medical University of Vienna (Table 1). Post-mortem time to freezing of the tissue varied from 6.5 to 30 h.

Primary antibodies

The following well-characterized primary antibodies are used: *Monoclonal (mouse) antibodies:* anti-(α)-ALDP (1D6 and 2B4, Euromedex, Souffel Weyersheim), α-GM130 (Golgi apparatus, Transduction Laboratories, Lexington, KY), α-protein disulfide isomerase (PDI, endoplasmic reticulum, Stressgen, Victoria, Canada), α-adrenocorticotropin (ACTH, DAKO, Carpinteria, CA), α-human luteinizing hormone (LH, DAKO), α-human follicle-stimulating hormone (FSH, DAKO), α-human thyroid-stimulating hormone (TSH, DAKO), α-PanCytokeratin Plus concentrated monoclonal antibody cocktail (panCK, DAKO), α-human neuron-specific enolase (NSE, DAKO), α-Vimentin (Vim, DAKO). *Polyclonal (rabbit) antibodies:* α-human growth hormone

Table 1
Human tissues investigated by immunohistochemistry and immunoblot

No.	Gen	Age	Diagnosis	Pit	ABr	FBr	Adr	Tes	Liv	Kid	Lun	Col	CaM	SkM	Ski	Thy	LyN	Pla
1	F	21	Metachromatic leukodystrophy	P					P	P								
2	M	86	Pneumonia	P			P	P										
3	M	59	Lung carcinoma, pneumonia	P, F			P, F	P										
4	F	78	Sepsis, cardiac failure	P, F			P, F											
5	F	85	Cardiac failure	P, F			P, F											
6	M	58	Pneumonia, sepsis	P, F														
7	F	62	Myocardial infarction	P, F			P, F									P		
8	M	64	Myocardial infarction, vascular encephalopathy	P, F	P			P	P	P	P	P	P			P		
9	M	49	Schizophrenia, cardiac failure	P, F														
10	F	78	Myocardial infarction	P, F														
11	M	64	Lung carcinoma	P, F														
12	F	10	Pneumonia [a]	P, F														
13	F	28	Pulmonary hypertension, LTX [a]	P, F			P, F											
14	F	63	Melanoma, neoplastic meningiosis [a]	P, F			P, F											
15	M	10	Glioblastoma [a]	P, F														
16	M	62	Glioblastoma [a]	P, F														
17	M	54	Sepsis, pneumonia				P, F	P										
18	F	71	Lung carcinoma						P	P	P	P	P	P			P	
19	F	81	Stenosis of aorta, cardiac failure						P	P	P	P	P	P	P		P	
20	M	53	Endocarditis						P	P	P	P	P	P	P		P	
21	F	38	Multiple sclerosis, sepsis						P	P	P		P					
22	M	66	Cardiomyopathy						P		P							
23	M	13	Myopathy												P			
24	M	49	Meningioma												P			
25	M	1.5	Glioma												P			
26	F	22	Lennox–Gastau syndrome		P													
27	M	47	Sepsis		P													
28	F	79	Lung carcinoma		P													
29	F	79	Pneumonia		P													
30	F	18 w	Trisomy 21			P				P								
31	M	22 w	Trisomy 13			P												
32	M	22 w	Spontaneous abortus			P				P								
33	M	16 w	Myotubular myopathy			P												
34	F	23 w	Complex vitium cordis			P				P								
35	F	19 w	Occipital encephalocele												P			
36	–	–	Data not available															P

P: paraffin material, investigated for immunohistochemistry; F: frozen material, investigated for Western blot; Gen: gender; Age: years, or w, weeks of gestation; LTX: lung transplantation; Pit: pituitary gland; ABr: adult brain; FBr: fetal brain; Adr: adrenal gland; Tes: testis; Liv: liver; Kid: kidney; Lun: lung; Col: colon; CaM: cardiac muscle; SkM: skeletal muscle; Ski: skin; Thy: thyroid; LyN: lymph node; Pla: placenta.

[a] Cortisol treatment longer than 10 days.

(hGH, DAKO), α-human prolactin (DAKO), α-neurofilament 68 kDa (neurons, axons; Chemicon, Temecula, CA), α-PMP70 (peroxisomal membrane protein 70 kDa, Affinity BioReagents, USA), α-cathepsin D (BioGenex, San Ramon, CA) and α-β-actin Mab1501 (Chemicon). *Polyclonal (sheep) antibody:* α-catalase (The Binding Site, Birmingham, UK).

Immunohistochemistry

Light microscopy

Immunohistochemistry of ALDP for light microscopy follows previously described protocols (Unterberger et al., 2006). Briefly, 2- to 4-μm-thick sections are deparaffinized and incubated in 3% hydrogen peroxide in methanol to block endogenous peroxidase activity. Slides are steamed in citrate buffer at pH 6.0 for antigen retrieval and incubated at 4 °C overnight with primary antibodies. A DAKO EnVision detection kit, peroxidase/DAB, rabbit/mouse (DakoCytomation, Glostrup, Denmark) is used for visualization of the antibody reaction. Negative controls are performed by omitting the primary antibody and by using Universal Negative Control rabbit (DAKO) for polyclonal rabbit antibodies or purified mouse myeloma IgG1 (Zymed Laboratories, San Francisco, CA) for monoclonal mouse antibodies.

Fluorescence labeling

Fluorescence labeling is performed as described in detail previously (Unterberger et al., 2006). The following well characterized fluorescent secondary antibodies are used: Alexa Fluor-488 coupled goat anti-mouse IgG (Molecular Probes, USA), Cy3 coupled donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), and Cy3 coupled streptavidin (Jackson ImmunoResearch). For double/triple labeling, appropriate combinations of primary and fluorescent secondary antibodies are used. In case of labeling with two monoclonal antibodies, the Zenon Alexa Fluor-546 coupled mouse IgG1 Labeling Kit (Molecular Probes, Leiden, The Netherlands) is used. The slides are analyzed with a Zeiss LSM 510 motorised confocal laser scan microscope (Carl Zeiss, Jena, Germany) equipped with an argon-ion laser source (488 nm excitation) and two HeNe lasers (543 and 633 nm excitation). To eliminate "bleed-through" from either channel, an appropriate combination of excitation and barrier filters (band-pass filter 505–550 nm and long-pass filters 560 nm and 650 nm) is used.

Quantification of labeled cells in the pituitary gland

ALDP-positive cells are counted in an area of 3 mm^2 in the anterior lobe of the pituitary gland. Standardised microscopic fields are defined by an ocular morphometric grid. Appropriate nonparametric tests are used for statistical analysis. *P*-values <0.05 are regarded as statistically significant.

Tissue blot analyses

A multiple-tissue expression array (MTE™) blot containing different human tissue samples and cell lines is purchased from Clontech. For detection of human *ABCD1* mRNA, a cDNA fragment corresponding to bp 661–1068 of the human ALDP cDNA sequence (GenBank Accession No. BC025358) is amplified by PCR from human liver cDNA using the following primers: 5′-TGCCGGAATTCGGGGCTGCTGGC-3′ (forward) and 5′-AGCCACGTCTAGAAGTGGCTTGG-3′ (reverse). The radioactive probe is generated by using the random priming method with [α-^{32}P] dCTP (Hartmann Analytic, Braunschweig, Germany) and HighPrime solution (Roche Diagnostics, Mannheim, Germany). As a control for equal loading and transfer, a 528-bp glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe prepared by PCR amplification from plasmid DNA using the primers 5′-ACCACCATGGAGAAGGCTGG-3′ (forward) and 5′-CTCAGTGTAGCCCAGGATGC-3′ (reverse) is used. The MTA blot is hybridized using Express Hyb solution (BD Bioscience, Franklin Lakes, NJ) at 65 °C and washed to high stringency according to the manu-

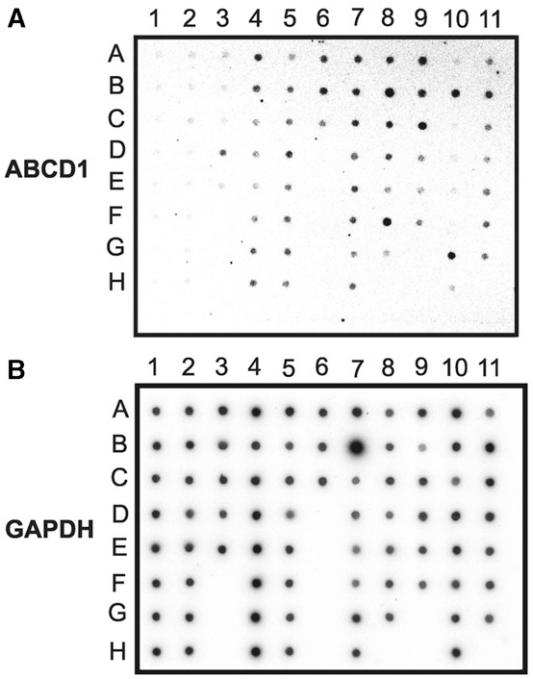


Fig. 1. Expression profile of *ABCD1* in various adult and fetal human tissues as well as different human cell lines. A commercially available multiple tissue expression array containing polyA$^+$ selected RNA from various human tissue samples and cell lines is hybridized with radiolabeled *ABCD1* cDNA (A) or *GAPDH* cDNA (B) as a control for equal loading and transfer. The tissue spots on the MTE™ RNA dot blot are the following: column 1: A, whole brain, B, cerebral cortex, C frontal lobe, D, parietal lobe, E, occipital lobe, F, temporal lobe, G, paracentral gyrus of cerebral cortex, H, pons; column 2: A, cerebellum left, B, cerebellum right, C, corpus callosum, D amygdala, E, caudate nucleus, F, hippocampus, G, medulla oblongata, H, putamen; column 3: A, substantia nigra, B, nucleus accumbens, C, thalamus, D pituitary gland, E spinal cord; column 4: A, heart, B, aorta, C, atrium left, D, atrium right, E, ventricle left, F, ventricle right, G, intraventricular septum, H, apex of the heart; column 5: A, esophagus, B, stomach, C, duodenum, D, jejunum, E, ileum, F, ileocecum, G, appendix, H, ascending colon; column 6: A, transverse colon, B, descending colon, C, rectum; column 7: A, kidney, B, skeletal muscle, C, spleen, D, thymus, E, peripheral blood leukocyte, F, lymph node, G bone marrow, H, trachea; column 8: A, lung, B, placenta, C, bladder, D, uterus, E, prostate, F, testis, G, ovary; column 9: A, liver, B, pancreas, C, adrenal gland, D, thyroid gland, E, salivary gland, F, mammary gland; column 10: A, leukemia HL-60 cells, B, HeLa S3 cells, C, leukemia K-562 cells, D, leukemia MOLT-4 cells, E, Burkitt's lymphoma Raji, F, Burkitt's lymphoma Daudi, G, colorectal adenocarcinoma SW480, H, lung carcinoma A549; column 11: A, fetal brain, B, fetal heart, C, fetal kidney, D, fetal liver, E, fetal spleen, F, fetal thymus, G, fetal lung.

facturer's instructions before autoradiographic exposure to a Kodak BioMax film (Kodak-Eastman, Rochester, NY, USA) or to a phosphoimager system (Bio-Rad).

Western blot analysis of pituitary and adrenal glands

At autopsy, pituitary and adrenal glands are separated into two halves. One half is fixed in formalin for immunohistochemical investigation. The other half is dissected into anterior/posterior lobe and cortex/medulla, respectively, and the pieces are frozen at −70 °C. Tissue is homogenized by using a potter in RIPA-buffer (50 mM TRIS pH 7.5, 500 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium-desoxycholate, 1% (w/v) NP-40) and after 20 min the extract is cleared by centrifugation (20,900×*g* for 15 min). Equal amounts of protein are subjected to Western blot analysis using anti-(α)-ALDP (1:1000), α-CK (1:1500), α-NSE (1:1000), α-vimentin (1:1000) and α-β-actin (1:10 000) antibodies. For reprobing membranes are washed with stripping buffer (0.2 M glycine, 0.5 M NaCl, pH 2.5) equilibrated with Tris-buffered saline with Tween 20 (TBS-T) (25 mM Tris pH 7.2, 150 mM NaCl and 0.05% (w/v) Tween-20) and then labeled with the next antibody.

Results

ABCD1 mRNA in human tissues

The gross distribution of *ABCD1* mRNA in human tissue samples and cell lines is investigated by multiple-tissue expression array (MTE) blot analysis. *ABCD1* mRNA is found in all examined tissues. Strong expression of *ABCD1* mRNA is detectable in pituitary gland, adrenal gland, testis, liver, kidney, digestive tract, cardiac and skeletal muscle, skin, placenta, and in fetal brain samples (Fig. 1). In contrast, the levels of *ABCD1* mRNA are only low in all selected samples of the adult central nervous system including entire adult brain, cerebral cortex, putamen, pallidum, caudate nucleus, cerebellum, and spinal cord.

Immunohistochemistry of ALDP in human tissues

Immunohistochemistry is performed to define the cellular and subcellular pattern of ALDP in various tissues of high *ABCD1* mRNA expression. In general, ALDP amounts markedly differ between different organs of one individual case and between the same organs of different cases.

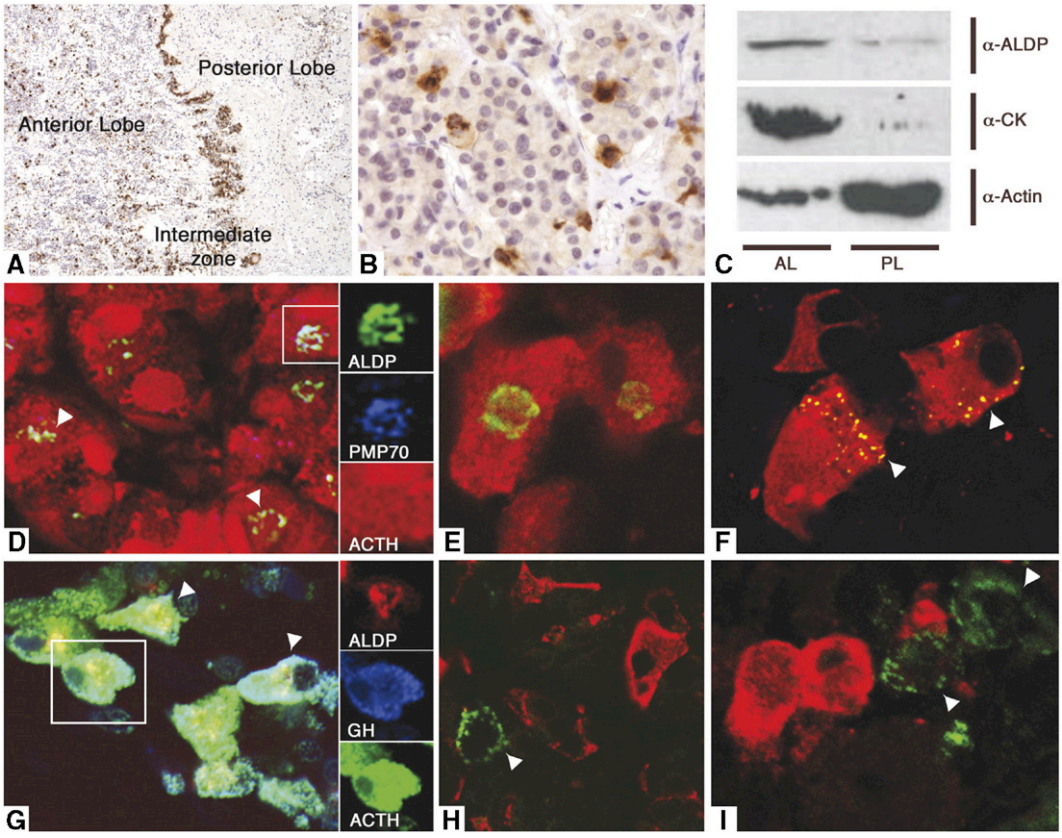


Fig. 2. Expression of ALDP in pituitary gland. Investigation of 16 pituitary glands reveals ALDP expression primarily in corticotroph cells of the adenohypophysis. Using immunohistochemistry, ALDP is found predominantly in the intermediate zone and to a lesser extent in the anterior lobe (A). ALDP is detectable in a subpopulation of endocrine cells (B). Immunohistochemical findings are substantiated by Western blot analysis, where ALDP is found predominantly in a protein extract generated from an anterior lobe sample (AL) rather than from a posterior lobe sample (PL) of the pituitary gland. The anterior lobe is identified by cytokeratin, which is not present in posterior lobe cells (C). Within ACTH-producing cells (red), ALDP (green) co-localizes with the peroxisomal protein PMP70 (blue, arrowheads) (D). ALDP is either arranged circularly near the nucleus (E), or evenly distributed throughout the cytoplasm (F) of ACTH-producing cells (ALDP green, ACTH red). Some of the ACTH and ALDP positive cells also stain for growth hormone (ALDP red, hGH blue, ACTH green) (G). In contrast, ALDP (green, arrowhead) is not found in cells exclusively containing prolactin (red) (H) or TSH (red) (I). Original magnification: (A) ×40; (B) ×400; (D, G, H and I) ×630; (E and F) ×1000.

Pituitary gland

In the pituitary gland ($n=16$), we find a specific pattern of regional and cellular distribution for ALDP with different proportions of positive cells in the anterior lobe (adenohypophysis), the intermediate zone, and the posterior lobe (neurohypophysis).

In the intermediate zone, almost every secretory cell contains ALDP, whereas within the anterior lobe ALDP is only confined to a subpopulation of secretory cells (Figs. 2A, B). In the posterior lobe, ALDP is generally absent, or only faint in some pituicytes and endothelial cells (data not shown).

Immunohistochemistry and immunofluorescence for ALDP shows a dot-like staining pattern and is colocalized with the peroxisomal proteins catalase and PMP70 (Fig. 2D). ALDP is distinctly separated from staining of lysosomes (cathepsin D), Golgi apparatus (GM130), and endoplasmatic reticulum (PDI) (data not shown). In general, the ALDP-positive peroxisomes are evenly distributed throughout the cytoplasm, although in six patients with very high ALDP levels a circular arrangement of ALDP-positive peroxisomes is visible near the nucleus of a proportion of cells (Figs. 2E, F). The perinuclear arrangement of ALDP in these tissues is most striking in the intermediate zone, whereas in the anterior lobe both even and circular distribution pattern can be observed to the same extent. Western blot analysis of anterior and posterior lobe tissue extracts (cytokeratin is used as marker for anterior lobe cells) confirms immunohistochemical findings, showing higher levels of ALDP in samples of anterior lobe than in the posterior lobe (Fig. 2C). For each lane, total amount of protein is indicated by β-actin.

Cellular pattern of ALDP in the pituitary gland

Confocal analysis reveals a strict colocalization of ALDP with ACTH-producing (corticotroph) cells (Figs. 2D–F). Some of the corticotroph cells co-express other hormones such as follicle-

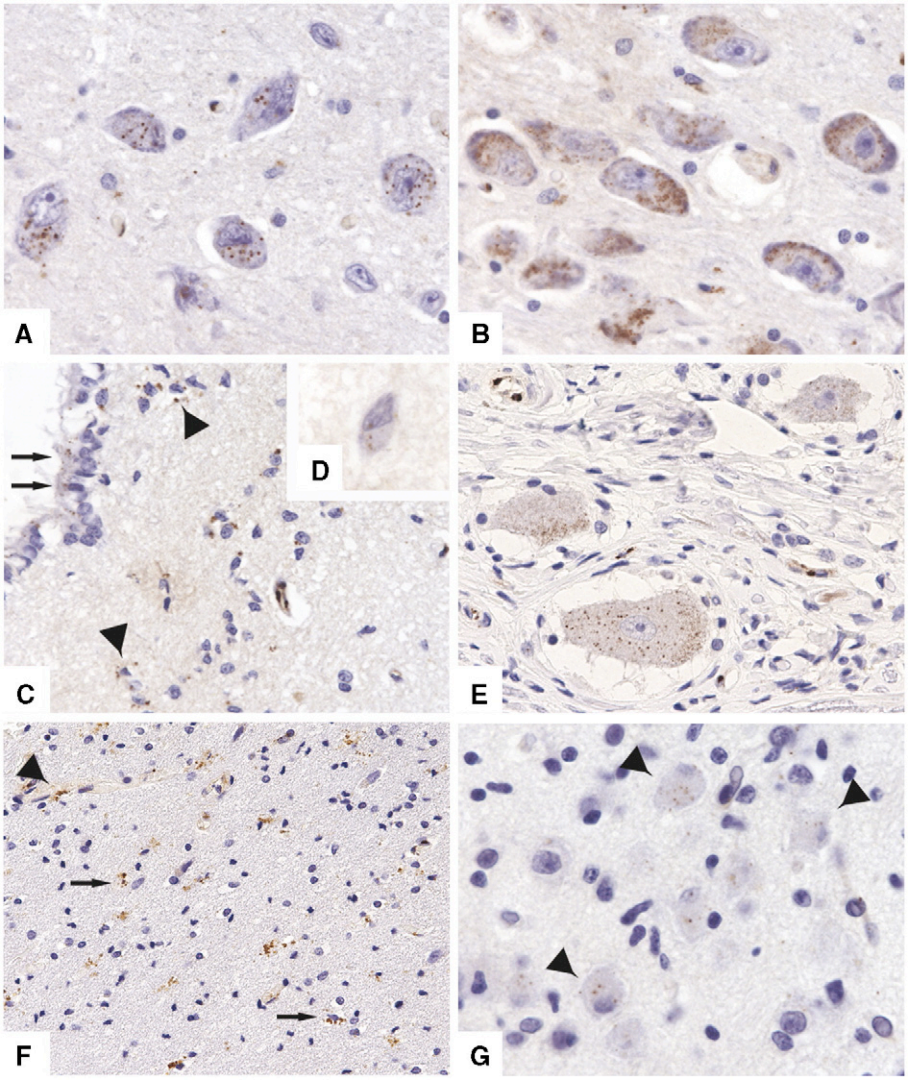


Fig. 3. Expression of ALDP in neurons. Immunohistochemical expression of ALDP is found in neurons of the hypothalamus (A), basal nucleus of Meynert (B) and periaqueductal grey matter in five adult brains (C, arrowhead, D). Additionally, some ALDP positivity is visible in the ependymal cell layer of the fourth ventricle (C, arrow). Neurons in the dorsal root ganglia of a 47-year-old male show high expression of ALDP (E). Endothelial cells (F, arrowhead) as well as a proportion of glial cells (F, arrows) are ALDP-positive in the subcortical white matter of five adult brains. Dot-like labeling of ALDP is observed in neurons in hypothalamus of a fetal brain in the 23rd week of gestation (G, arrowhead). Original magnification: (A–C, and G) ×400; (D) ×600; (E and F) ×200.

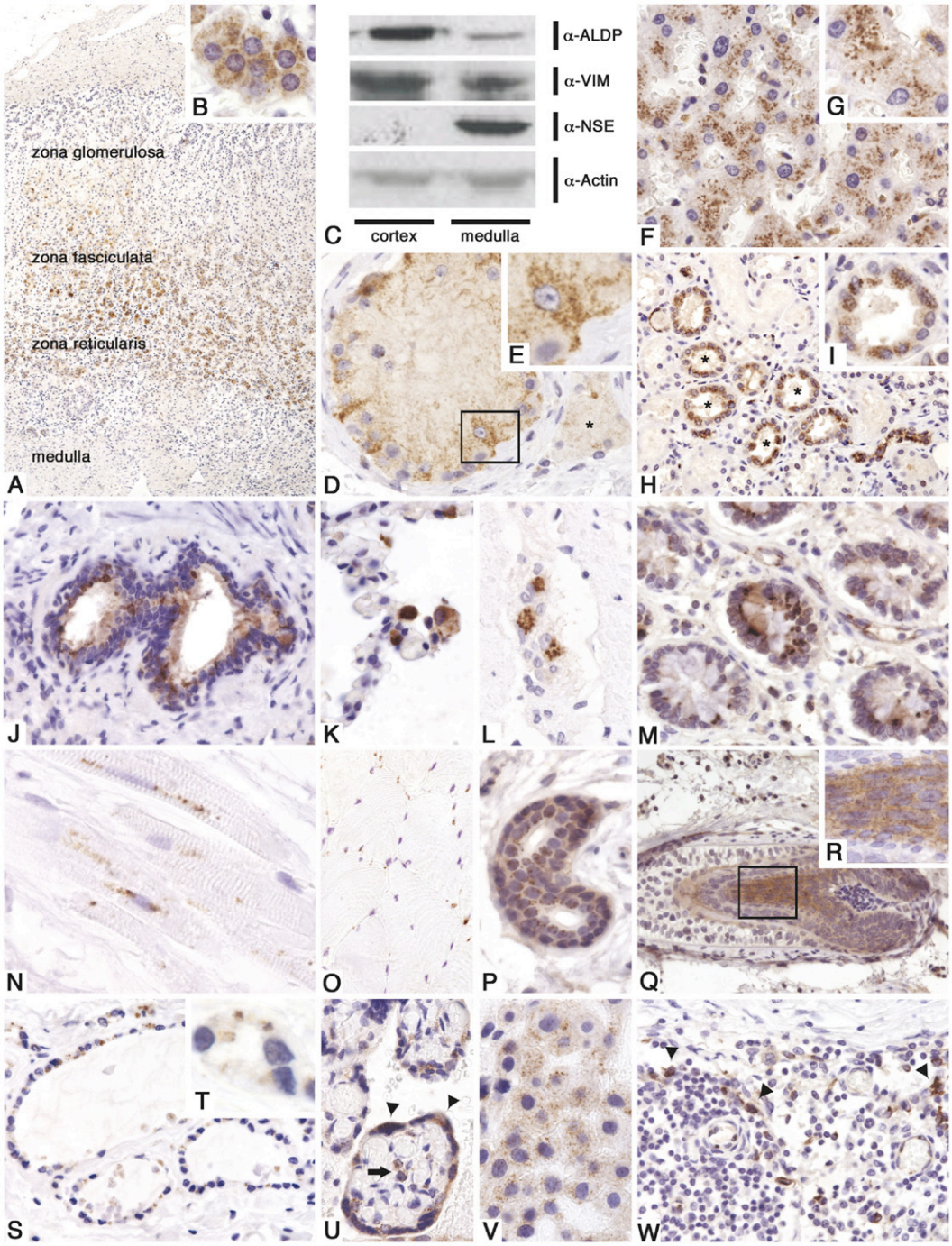
stimulating hormone (FSH), luteinizing hormone (LH), prolactin, or human growth hormone (hGH; Fig. 2G). However, no ALDP can be found in cells exclusively producing thyroid-stimulating hormone (TSH), FSH, LH, hGH or prolactin. (Figs. 2H, I).

Interestingly, pituitary glands from patients who received long-term cortisol treatment *ante exitum* ($n=5$) show fewer ACTH-positive cells and also contain a significantly reduced number of ALDP-positive cells (mean, 28.5 cells/mm^2; standard error, 15.0; $p<0.002$) compared to patients without cortisol treatment ($n=11$; mean, 193.5 cells/mm^2; standard error, 23.5).

Moreover, ALDP is not detectable in tumour cells in three biopsies of corticotroph adenomas (data not shown).

Adult and fetal brain

Within grey and white matter of five adult brains, a specific cellular and regional pattern of ALDP expression can be found. A marked number of neurons of the hypothalamus, basal nucleus of Meynert, periaqueductal grey matter and in the area of locus coeruleus express ALDP (Figs. 3A–D). In contrast, neuronal ALDP expression is only moderate in thalamus and dorsal nucleus of vagus, sparse in frontal, temporal, parietal and occipital cortex, hippocampus and amygdala, and absent in the putamen, caudate, subthalamic, dentate, and olivary nuclei, and substantia nigra and in neurons of the cerebellum (data not shown). In one thoracal dorsal root ganglia sample available, about 40% of neurons show



positivity for ALDP (Fig. 3E). Moreover, ALDP is found in endothelial cells, the entire ependymal cell layer of the ventricular system (Fig. 3C), choroid plexus and in Bergmann glia in four out of five cerebellum samples. Astrocytes and microglia reveal marked ALDP positivity in the subcortical (Fig. 3F) and cerebellar white matter but are mostly negative in long fiber tracts such as the internal capsules, corpus callosum, lemniscus medialis, and the corticospinal tract. ALDP intensity in oligodendrocytes is much lower and can only be detected in a small proportion of cells localized in the subcortical and cerebellar white matter. In five fetal brains (Table 1), the pattern of ALDP expression is similar to that of adult brains, showing ALDP in endothelial and ependymal cells, choroid plexus and neurons of the hypothalamus (Fig. 3E).

Other tissues

Cellular distribution of ALDP is investigated in adrenal gland ($n=8$), testis ($n=4$), colon ($n=4$), kidney (adult, $n=6$; fetal, $n=3$), liver ($n=7$), skin (adult, $n=5$; fetal $n=1$), cardiac ($n=5$) and skeletal muscle ($n=3$), lung ($n=6$), thyroid ($n=2$), placenta ($n=1$) and lymph node ($n=3$).

Connective tissue. ALDP can be found in cellular constituents of the stroma of all investigated organs including endothelial cells, macrophages, and proliferating fibroblasts.

Adrenal gland. The three concentric zones of the adrenal cortex show different expression of ALDP, with highest amounts in the zona fasciculata and zona reticularis. Apart from vascular stroma the adrenal medulla reveals no ALDP-positive peroxisomes (Fig. 4A). Western blot analysis of adrenal medulla and cortex tissue extracts (neuron specific enolase and vimentin are used as marker for medulla and cortex) confirm that ALDP levels are higher in the cortex than in medulla of the adrenal gland (Fig. 4B). The relative amount of protein is indicated by β-actin. Adrenal glands from two patients who received long-term cortisol treatment *ante exitum* show lower amounts of ALDP in the adrenal cortex compared to six patients without cortisol treatment.

Testis. Dot-like labeling of ALDP in varying amounts can be found in Sertoli cells and Leydig cells in the interstitium (Figs. 4D, E). Interestingly, exceptionally high quantity of ALDP is detectable in Sertoli cells of a patient with germ cell aplasia due to repeated cycles of chemotherapy for lung carcinoma.

The ALDP expression pattern in liver, kidney, lung, colon, cardiac and skeletal muscle, skin, thyroid, placenta, and lymph node is described and summarized in Table 2.

Discussion

In this study, we investigate the expression of *ABCD1* in a broad range of human tissues and for the first time systematically describe the distribution of ALDP in different cell types of the human body. The human *ABCD1* expression is most abundant in adrenal gland, testis, liver, kidney, lung, placenta, and intestine. Overall, these findings are in good agreement with the previously described expression profile (Langmann et al., 2003) with the exception of kidney, liver, and adrenal gland which we find to be major sites of *ABCD1* expression. These data are supported by the immunohistochemical analysis where we detect high amounts of ALDP in hepatocytes, distal tubules, and adrenocortical cells. Moreover, comparisons with expression data obtained for human (Mosser et al., 1993) and mouse tissues (Berger et al., 1999; Lombard-Platet et al., 1996; Mutch et al., 2004; Troffer-Charlier et al., 1998) reveal no major differences. In addition, we find high *ABCD1* mRNA levels in the pituitary gland and intermediate expression levels in a variety of other tissues, such as fetal organs, lymph node or stomach that have not been investigated until now (Fig. 1). Overall, very high expression of *ABCD1* is obtained in tissues that have high energy requirements such as heart, muscle, liver and the renal and endocrine systems. Interestingly, adrenal cortex and testis are the only extra-neural tissues severely affected in X-ALD (Brennemann et al., 1997; Korenke et al., 1997; Powers and Schaumburg, 1981). This indicates that the level of *ABCD1* expression does not correlate with the site of pathology and clinical symptoms of X-ALD patients. This discrepancy might be caused either (I) by compensatory effects exerted by other ABC-transporter proteins (ALDRP, PMP70 or PMP69) solely in non affected tissues, (II) by sensitivities of different tissues towards a deficiency in ALDP or the associated sequelae, or (III) because the actual substrate for ALDP is different from VLCFAs, and this unknown substrate is highly abundant or toxic only in disease associated tissues. This would also indicate that defective peroxisomal β-oxidation is not the underlying cause of X-ALD associated pathology (Berger and Gartner, 2006). The latter hypothesis is further supported by the fact that the clinical phenotype of patients with acyl-CoA oxidase deficiency (the first step of peroxisomal VLCFA β-oxidation) (Watkins et al., 1995) is more severe than the two main clinical presentations found in X-ALD patients (cerebral ALD and adrenomyeloneuropathy).

Analysis of ALDP distribution confirms the expression pattern of *ABCD1* mRNA at the protein level and discloses differences between organ-specific cell types. ALDP selectively occurs in specific cell types of kidney (distal tubules), skin (eccrine gland, hair

Fig. 4. Expression of ALDP in human tissues. Expression of ALDP in eight adrenal glands is investigated by immunohistochemistry (A adrenal gland, B zona reticularis cells of adrenal cortex) and Western blot analysis (C; NSE: neuron specific enolase as marker for medulla, Vim: vimentin expressed in both cortex and medulla). ALDP is found in high amounts in adrenal cortex but is very low in adrenal medulla. Immunohistochemical examination of four testis samples detects ALDP in Leydig cells (D, asterisk) and Sertoli cells (D, rectangle enlarged in panel E). ALDP is strongly stained in hepatocytes of seven liver samples (F, G). High levels of ALDP are found in distal tubule cells in six investigated kidneys (H, asterisk, I distal tubule in detail). ALDP is prominent in ciliated cells lining the airway of a 71-year-old female with chronic obstructive pulmonary disease (J) and is constantly visible in pulmonary macrophages including alveolar macrophages of all six lungs investigated (K). Strong ALDP immunoreactivity is visible in ganglion cells of the neural plexus in the submucosa and muscularis propria of four colon samples (L). In addition, ALDP is found in endothelial cells and macrophages in the connective tissue of lamina propria as well as columnar epithelium lining the colonic crypts (M). In myofibers, ALDP positive peroxisomes are distributed mainly in the center of the fiber in cardiac muscle ($n=5$) (N) or in perinuclear regions beneath the sarcolemma in skeletal muscle ($n=3$) (O). In five skin biopsy and autopsy samples, highest amount of ALDP is detectable in the excretory component of the eccrine gland (P) and in the hair medulla of the hair follicle (Q, rectangle enlarged in panel R). Slight staining of ALDP is shown in cuboidal cells in thyroid of a 64-year-old male (S, T). In one placenta, ALDP is found in syncytiotrophoblast, to a lesser extent in the cytotrophoblast, and in macrophages (U syncytiotrophoblast, arrowheads; macrophages, arrow). In addition, ALDP is visible in decidua cells (V). In lymph node ($n=3$), ALDP is most abundant in macrophages in the sub-capsular and medullary sinuses (W macrophages in sub-capsular sinus, arrowheads). Original magnification: (A) ×40; (B, D–G, I–N, and S–W) ×400; (H and Q) ×200; (O, P, and R) ×600.

Table 2
Cellular localization of ALDP in human tissues

Tissue	Fig. 4	Amount of ALDP mRNA	Cellular localization	Amount of ALDP
Liver ($n=7$)	F, G	+++	Hepatocytes	+++
			Macrophages (Kupffer cells)	+/++
Kidney (adult, $n=6$; fetal, $n=3$)	H, I	Adult: +++	Distal tubule (including Henle's loop and distal convoluted tubule)	Adult and fetal: +++
		Fetal: ++	Proximal tubule	Adult and fetal: −
			Collecting duct	Adult: − Fetal: +++
Lung ($n=6$)	J, K	+++	Macrophages	+/++
			Ciliated cells of airways	Variable (+/−, +++)
Colon ($n=4$)	L, M	+++	Ganglion cells of plexus submucosus and myentericus	+++
			Columnar epithelium and endocrine cells (specified by Grimelius silver impregnation)	Variable (+/−, +++)
Cardiac ($n=5$)/skeletal muscle ($n=3$)	N, O	+++	Myofibers	++
Skin (adult, $n=5$; fetal, $n=1$)	P, Q, R	n.d.	Medulla of hair follicle	Adult and fetal: +++
			Excretory component of the eccrine gland	Adult: ++ Fetal: −
			Secretory portion of the eccrine gland	Adult: +/− Fetal: −
			Sebaceous gland	Adult and fetal: −
			Basal layer of stratified squamous epithelium	Adult and fetal: ++
			Arrector pili-muscle	Adult and fetal: +
			Adipocytes	Adult: +/− Fetal: n.d.
Thyroid ($n=2$)	S, T	++	Cuboidal (secretory active) follicular cells	+
			Flattened (relatively inactive) follicular cells	−
Placenta ($n=1$)	U, V	+++	Syncytiotrophoblast	+++
			Cytotrophoblast	+
			Decidua cells	++
			Macrophages (Hofbauer cells)	+++
Lymph node ($n=3$)	W	++	Macrophages in sub-capsular and medullary sinuses	++
			Lymphocytes	−
			Antigen presenting cells	+/−

+++: marked, ++: moderate, and +: slight positivity of ALDP, +/−: most cells are negative or show very little ALDP reactivity, −: ALDP is virtually absent; variable: ALDP varied from one case to another, with some cells strongly ALDP-positive in one case and almost absent ALDP reactivity in another case, n.d.: not done.

follicles and fibroblasts), colon (ganglion cells and epithelium), adrenal gland (zona reticularis and fasciculata), testis (Sertoli and Leydig cells) and pituitary gland (corticotroph cells). Moreover, we find examples of ALDP up-regulation upon functional activation of individual cell types. Secretorily active follicular cells in the thyroid contain more ALDP than inactive ones, and also macrophages show more ALDP than monocytes. These results are in good agreement with increased ALDP levels in remyelinating oligodendrocytes and reactive astrocytes (Fouquet et al., 1997). In this context, the high ALDP level in Sertoli cells of a patient having received chemotherapy might also indicate an elevated activation state during recovery from chemotherapy-induced damage.

In brain, we analyze the expression of ALDP in a wide range of anatomical regions, focussing on subcortical nuclei. In the CNS white matter, ALDP is visible in subcortical and cerebellar white matter, which is in good agreement with prior studies that were performed in these areas (Fouquet et al., 1997). However, commissural fibers such as the corticospinal tract, lemniscus medialis, or corpus callosum reveal very little ALDP in human brain but were described to be high in mouse brain. High levels of immunohistochemically detectable ALDP are found in dorsal root ganglia, which is of particular interest, since it was shown that these cells undergo atrophy in patients with AMN (Powers et al., 2001). Moreover, hypothalamus and basal nuclei of Meynert are strongly ALDP-positive, although this is not mirrored by the overall expression of *ABCD1* in brain, which appears low in the MTE blot. This discrepancy might be explained by the lack of these selected areas in the commercially available RNA blot array. Interestingly, *ABCD1* mRNA level of fetal brain is remarkably higher than of adult brain. This may be due to developmental changes in *ABCD1* expression, or because the fraction of fetal brain volume occupied by hypothalamus and thalamus as areas of high *ABCD1* expression is larger than in the adult brain.

One of the main characteristics shared by all areas of high ALDP expression in brain (hypothalamus, basal nucleus of Meynert, periaqueductal grey matter and locus coeruleus) is the occurrence of peptide hormones that are derived from the precursor proopiomelanocortin (POMC) (Forslin Aronsson et al., 2006; Göktalay et al., 2006; Hadley and Haskell-Luevano, 1999; Reyes et al., 2006; Sukhov et al., 1995). This might indicate that POMC-derived peptide hormones are a common feature of cells with high ALDP expression, and is further corroborated in the pituitary gland where ALDP is found in ACTH producing cells and not in cells that produce peptide hormones absent ACTH. In the hypothalamo–pituitary–adrenal axis, ACTH is known to regulate cells of the adrenal zona fasciculata that secrete glucocorticoids, which act on the pituitary gland exerting a negative feedback. Interestingly, in our study, glucocorticoids do not only suppress ACTH synthesis by down-regulating the expression of its precursor POMC (Hadley and Haskell-Luevano, 1999), but are also found to reduce ALDP levels in pituitary and adrenocortical cells of patients receiving cortisol treatment for a longer period (>10 days) *ante exitum*. This co-regulation supports the important role of ALDP in the hypothalamo–pituitary–adrenal endocrine system and suggests a functional

correlation between ALDP and POMC-derived hormones such as ACTH. The functional significance of ALDP in this context is still unclear. In X-ALD patients, it is presumed that the adrenal gland fails to produce sufficient glucocorticoids due to dysfunction and apoptotic death of adrenocortical cells, which secondarily increases the levels of ACTH. Thus, a requirement of ALDP for ACTH synthesis is unlikely since high levels of ACTH due to the primary adrenal insufficiency are found in X-ALD patients (Korenke et al., 1997; Powers et al., 1980). The absence of ALDP in corticotroph cell adenomas would allow the assumption that ALDP is related to a normal function of the corticotroph cells such as sudden and frequent changes in the functional demand on hormonal synthesis, secretion, recycling, storage or release made upon these hormonal cell types (Horvath et al., 1977). Finally, it cannot be excluded that ALDP and ACTH might act independently from each other but share a currently unspecified physiological process.

In conclusion, we find high ALDP levels in cells characterized by steroid hormone production (e.g. adrenal gland and testis) and POMC-derived peptide hormone synthesis (e.g. pituitary gland, basal nuclei of Meynert or hypothalamus). Thus, ALDP might have an additional connection to peptide hormones comparable to the already described association with steroid hormones (Brennemann et al., 1997; Korenke et al., 1997), explaining disturbances in the hypothalamo–pituitary–adrenal endocrine system in X-ALD patients.

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