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Differential Aminoacylase Expression in Neuroblastoma

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Abstract

Neuroblastoma, a cancer of the sympathetic nervous system, is the most common extracranial solid tumor in children. MYCN amplification and increased BDNF/TrkB signaling are features of high-risk tumors; yet, only ~25% of malignant tumors display these features. Thus, the identification of additional biomarkers and therapeutic targets is essential. Since aminoacylase 1 (ACY1), an amino acid deacetylase, is a putative tumor suppressor in small cell lung and renal cell carcinomas, we investigated whether it or the other family members aspartoacylase (ASPA, aminoacylase 2) or aminoacylase 3 (ACY3) could serve a similar function in neuroblastoma.

Aminoacylase expression was examined in TrkB-positive, MYCN-amplified (SMS-KCNR and SK-N-BE) and TrkB-negative, non-MYCN amplified (SK-N-AS, SK-N-SH, SH-SY5Y, and SH-EP) neuroblastoma cell lines. Each aminoacylase exhibited distinct spatial localization (i.e., cytosolic ACY1, membrane-associated ASPA, and nuclear ACY3). When SK-N-SH cells were treated with neural differentiation agents (e.g., retinoic acid, cAMP) in media containing 10% serum ACY1 was the only aminoacylase whose expression was up-regulated. ASPA was primarily expressed in SH-EP cells of a glial sublineage. ACY3 was more highly expressed in the TrkB-positive, MYCN-amplified lines. All three aminoacylases were expressed in normal human adrenal gland, a common site of neuroblastoma origin, but only ACY1 and ACY3 displayed detectable expression in primary neuroblastoma tumor. Bioinformatics data mining of Kaplan-Meier survival revealed that high ACY3 expression is correlated with poor prognosis; while, low expression of ACY1 or ASPA is correlated with poor prognosis. These data suggest that aminoacylase expression is dysregulated in neuroblastoma.

Neuroblastoma, a cancer of the sympathetic nervous system, is the most common extracranial solid tumor in children.[1] Tumor cells likely emerge from the sympatho-adrenal lineage of the autonomic nervous system, with the majority of tumors forming in the adrenal medulla and the paraspinal sympathetic ganglia. Complete tumor regression occurs in a subset of infants with seemingly advanced cases.[2] Unfortunately, most advanced stage neuroblastomas are fatal because they are widely metastatic or refractory to therapy. High-risk tumors frequently exhibit amplification of the proto-oncogene MYCN[3] and expression of the TrkB neurotrophin receptor and its conjugate ligand, brain-derived neurotrophic factor (BDNF).[4, 5] However, despite advances in tumor characterization, only a fraction of

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malignancies display such features. Identifying novel molecular markers is essential toward advancing our understanding of the etiology of neuroblastoma as well as developing appropriate clinical interventions.

N-acetylation protects proteins from proteolytic degradation by aminopeptidases present in eukaryotic cells, thus increasing their half-life. Subsequent to cleavage by intracellular proteases (e.g., cathepsins, calpains, proteasomes, metalloproteinases), the released NH_2-blocked dipeptide is catabolized by acylpeptide hydrolase to release an NH_2-α-amino acid which undergoes deacetylation by an aminoacylase.[6] Aminoacylases are cytosolic, zinc-dependent metalloenzymes responsible for catalyzing the deacetylation of N-acyl-L-amino acids to generate acetate and a free amino acid, which then is available for protein synthesis.[7] Aminoacylase 1 (ACY1), the most widely expressed aminoacylase (e.g., kidney, liver, pancreas, spleen, thymus, brain, lung, and heart and skeletal muscle), is involved in the hydrolysis of a wide range of N-acetylated amino acids.[8] ACY1 serves as a putative tumor suppressor in renal cell carcinoma[9] and small cell lung cancer.[10] Aspartoacylase (ASPA, aminoacylase 2), a central nervous system (CNS) glial protein, is best known for its role in myelin lipid synthesis via deacetylation of neuronally derived N-acetyl-L-aspartate (NAA).[11] Missense mutations in ASPA result in Canavan disease, a fatal childhood leukodystrophy characterized by dysmyelination and spongi-form degeneration of white matter.[12] Aminoacylase 3 (ACY3), which is primarily expressed in liver and kidney, is the least understood family member because it deacetylates N-acetylated aromatic amino acids, which are present at relatively low frequencies in eukaryotic proteins, and mercapturic acids.[13, 14] Given the putative tumor suppressor role of ACY1, we sought to characterize aminoacylase expression in neuroblastoma.

We report, for the first time, aminoacylase expression in six well-characterized neuroblastoma cell lines (i.e., TrkB-positive, MYCN-amplified SMS-KCNR and SK-N-BE and TrkB-negative, non-MYCN-amplified SK-N-AS, SK-N-SH, SH-SY5Y, and SH-EP). In addition to differential aminoacylase expression in these cell lines under basal conditions and in response to neural differentiation agents, we characterized the expression pattern in normal human adrenal gland and neuroblastoma tumors. Each aminoacylase displayed discrete expression patterns suggesting each subserves distinct functions.

Materials and Methods

Cell Culture

Aminoacylase expression was examined in well-characterized neuroblastoma cell lines derived from the bone marrow of patients whose disease was refractory to treatment. The SK-N-SH line, obtained from a 4-year-old female, possesses many properties of neural crest cells.[15] This TrkB-negative line is composed of at least two different cell types and was subcloned into neuroblast-like SH-SY5Y and melanocytic/Schwann cell-like SH-EP lines. SK-N-AS cells, isolated from a 6-year-old female, are also TrkB-negative. Both SK-N-SH and SK-N-AS cells lack MYCN amplification. In contrast, the SMS-KCNR line, derived from an 11-month-old male[16], and the SK-N-BE(2) line (hereafter referred to as SK-N-BE), obtained from a 2-year-old male[17], produce endogenous BDNF[18] and are both TrkB-positive[18] and MYCN-amplified[17].

All cell lines were incubated in RPMI 1640 medium containing 2.05 mM glutamine (Mediatech; Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone; Logan UT), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen; Carlsbad, CA). Cells were plated at a density of 10,000 cells per well of 24-well plate or 20,000 cells/cm^2 per 6-cm dish, media was replenished every 48 hours, and cells were harvested after 1, 2, 3, and 5 days.

Differentiation assays were performed as previously described.[19, 20] SK-N-SH and SMS-KCNR cells were plated at a density of 20,000 cells per well of 24-well plate or 10,000 cells/cm^2 per 6-cm dish. Twenty-four hours after plating, cells were incubated in growth media containing one of the following differentiation agents: all-trans retinoic acid (ATRA, 10 μM; Sigma; St. Louis, MO), dibutyryl adenosine 3′, 5′-cyclic monophosphate (cAMP, 1 mM; Sigma), or forskolin (1 μM; Sigma) to induce neuronal differentiation; or 5-bromo-2-deoxyuridine (BrdU, 10 μM; Sigma) to induce Schwannian differentiation. In a second set of experiments, 24 hours after plating, cells were growth arrested in media containing 1% FBS for 12 hours prior to incubation with ATRA (5 μM) or cAMP (1 mM). Culture media and differentiation agents were replenished every 48 hours and cells were harvested 7 days after differentiation induction.

Western Blot Analysis

SDS-PAGE (25 μg protein from whole cell lysates) and western blotting was performed as previously described[21] using rabbit anti-human ACY1 (1,000X; Strategic Diagnostic Inc., Newark, DE), rabbit anti-human ASPA (1250X; sc-98734; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-mouse ACY3 (1,000X, kindly provided by Dr. Alexander Pushkin, Dept. of Medicine UCLA Medical School)[13], mouse anti-human β-III tubulin (2,500X; ab7751; Abcam, Cambridge, MA), and goat anti-human actin (1,000X; sc-1616; Santa Cruz Biotech.) primary antibodies in conjunction with species-specific HRP-conjugated (3,000X) secondary antibodies (Jackson ImmunoResearch; West Grove, PA). Immunocomplexes were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA) and densitometry performed using Quantity One software (Bio-Rad; Hercules, CA).

Fluorescence Microscopy

Immunocytochemistry was performed as previously described[21] using rabbit anti-human ACY1 (100X; Strategic Diagnostic), rabbit anti-mouse ASPA (1,500X, kindly provided by Dr. Aryan M.A. Namboodiri, Dept. of Anatomy, Physiology & Genetics USUHS)[22] and chicken anti-mouse ACY3 (100X, kindly provided by Dr. Alexander Pushkin) primary antibodies in conjunction with species-specific Cy3-conjugated (500X) secondary antibodies (Jackson ImmunoResearch). Blocking experiments were performed by incubating primary antibodies with a 5-fold molar excess of recombinant protein, purified from stably transfected HOG cells, overnight at 4 °C with shaking prior to immunocytochemistry as described above.

Immunohistochemistry, optimized to reduce auto-fluorescence, was performed on human tissue arrays (AD2081t and T433; US Biomax, Inc., Rockville, MD) as previously described.[23, 24] Briefly, slides were deparaffinized, subjected to antigen retrieval (Dako Inc., Carpinteria, CA) for 30 minutes, cooled in antigen retrieval solution for 15 minutes, and rinsed with 1% BSA/PBS for 5 minutes prior to incubation with 0.1% Sudan Black B (Sigma) in 70% methanol for 20 minutes. After washing three times for five minutes in 1% BSA/PBS containing 0.02% Tween and blocking for 45 minutes in PBS with 1% BSA, 0.02% Tween, and 10% goat serum, primary antibody was applied overnight. Slides were washed with 1% BSA/PBS, incubated with secondary antibody (in 1% BSA/PBS) for 45 minutes, washed as before, and cover slipped with Citifluor (Electron Microscopy Sciences; Hatfield, PA). Immunoreactivity was visualized with a Nikon Eclipse 800 epifluorescence microscope (MicroVideo Instruments; Avon, MA), and digital images captured with a SPOT RT digital camera (Diagnostic Instruments; Sterling Heights, MI).

Statistics

All data are from at least three independently prepared cultures and are expressed as mean ± standard error of the mean (SEM). Significant interactions were determined using one-way

ANOVA and Bonferroni's multiple comparison tests on Prism software (GraphPad; San Diego, CA). Statistical significance was assigned at probability of $p < 0.05$.

Results

Aminoacylases are differentially expressed in neuroblastoma cell lines

Western blot analysis revealed differential aminoacylase expression in the six neuroblastoma cell lines examined. With minor exceptions (i.e., ACY1 in SK-N-AS, ASPA in SMS-KCNR, and ACY3 in SH-EP cells) aminoacylase expression was unaltered by time in culture; thus expression appears unaffected by confluency. In contrast to what one might predict for a tumor suppressor, one-way ANOVA revealed greater total ACY1 expression in MYCN-amplified SMS-KCNR cells than non-MYCN-amplified SK-N-SH cells ($p = 0.011$) (Fig. 1A). Two immunoreactive species were identified, the predicted 50 kDa monomer and a 65 kDa oligomer. The 50 kDa protein was decreased in SK-N-BE cells ($p = 0.004$) while the 65 kDa species was decreased in SK-N-SH cells ($p < 0.0001$) relative to all other lines. In contrast to ACY1, one-way ANOVA revealed greater total ASPA expression in SK-N-SH cells and least in SMS-KCNR cells ($p < 0.0001$) (Fig. 1B). Both the predicted 36 kDa monomer and a 71 kDa dimer showed similarly regulated expression. Total ACY3 expression did not differ among the cell lines ($p = 0.80$), due to the fact that the predicted 35 kDa monomer, like ASPA, was more highly expressed in non-MYCN-amplified lines ($p < 0.0001$), while the 80 kDa dimer, like ACY1, was more highly expressed in MYCN-amplified lines ($p = 0.003$) (Fig. 1C). Aminoacylase antibody specificity was verified by western blot analysis against ACY1, ASPA, and ACY3 recombinant protein produced in stable tet-inducible cells. No cross-reactivity was observed (Fig. 1D).

Immunocytochemical analysis revealed differential aminoacylase subcellular localization (Fig. 1E). ACY1 displayed cytoplasmic staining in SMS-KCNR and SK-N-AS cells (Fig. $1E_{A, G}$), and more centriolar localization in SK-N-BE and SK-N-SH cells and subclones (Fig. $1E_{D, J, M, P}$). ASPA immunoreactivity was nearly absent in neuroblastic cells (Fig. $1E_{B, E, H, N}$), but displayed punctate, membrane-associated expression in Schwannian cells in the SK-N-SH and SH-EP lines (Fig. $1E_{K, Q}$). In contrast to ACY1 and ASPA, ACY3 expression showed similar nuclear subcellular localization in all lines (Fig. $1E_{C, F, I, L, O, R}$). Immunofluorescence specificity was demonstrated by blocking with recombinant protein produced in stable tet-inducible cells (Fig. $1E_{S, T, U}$), pre-incubation with immunizing peptide (Fig. $1E_X$), and exclusion of primary antibody (Fig $1E_{V, W}$). Taken together, the western blot and immunocytochemical analyses identified distinct trends in aminoacylase expression suggesting a unique regulation for each aminoacylase in neuroblastoma.

Induction of neuroblastoma differentiation alters aminoacylase expression

Neuroblastoma tumors are histologically very diverse with some tumors demonstrating a bipotential capacity to differentiate toward neuronal and glial lineages, with those tumors possessing abundant Schwannian stroma being more histologically and clinically favorable.[25] Thus, we next sought to investigate whether differentiation of neuroblastoma cells would alter aminoacylase expression. Of the cell lines used in this study, SK-N-SH cells exhibit the greatest heterogeneity, with cells evincing either a small neuroblastic or a larger Schwannian morphology.[15] Conversely, SMS-KCNR cells are exclusively neuroblastic in appearance. The regulation of aminoacylase expression was examined in SK-N-SH and SMS-KCNR cells treated with all trans-retinoic acid (ATRA), dibutyryl cAMP (cAMP), or forskolin to induce neuronal differentiation or with BrdU to induce Schwannian differentiation.[19] Because the neural differentiation agents may not be able to override the mitogenic signals present in the medium (i.e., 10% FBS), additional SK-N-SH cells were

growth arrested (i.e., grown in medium containing 1% FBS for 12 hours) prior to differentiation induction.

Western blot analysis of cells after 7 days in differentiation media revealed that the expression of only one aminoacylase, ACY1, was altered by differentiation (Fig. 2). More specifically, expression of the 65 kDa isoform, but not the 50 kDa monomer, was significantly up-regulated in SK-N-SH cells in response to all differentiation agents, even in the presence of 10% FBS (Fig. 2A). In sharp contrast, ACY1 expression was down-regulated in SMS-KCNR cells in response to differentiation. Neither ASPA (Fig. 2B) nor ACY3 (Fig. 2C) expression was altered in either SK-N-SH or SMS-KCNR cells upon differentiation. βIII-tubulin expression was used as a marker to confirm neuronal differentiation (Fig. 2D lanes 2–4 relative to lane 1, lanes 11 and 12 relative lane 10). Note that the basal level of βIII-tubulin expression is greater in SMS-KCNR line which primarily possesses neuroblastic cells (Fig. 2D, lane 6) than the heterogeneous SK-N-SH line (Fig. 2D, lane 1) that possesses both neuroblastic and Schwannian cells. BrdU treated SMS-KCNR cells (Fig. 2D, lane 9) showed reduced βIII-tubulin expression (Fig. 2D, lane 6), either due to Schwannian differentiation, which was not apparent immunocytochemically, or, more likely, due to growth arrest.

Since western blot analysis fails to take into account cellular heterogeneity (e.g., loss of Schwannian expression counter-balanced by an equivalent gain of neuronal expression), the spatial distribution of aminoacylase expression was examined immunocytochemically (Fig. 3). Morphological differences were apparent in SK-N-SH cells even when differentiated in media containing 10% FBS (Figs. $3A_{D, H, L, P}$). Centriolar ACY1 expression (Fig. $3A_{A}$) was lost upon neuronal (Figs. $3A_{E, I}$) and Schwannian differentiation (Fig. $3A_{M}$). ASPA expression was largely unaltered by differentiation (Figs. $3A_{B, F, N}$), with the exception of decreased expression with cAMP treatment (Fig. $3A_{J}$). In contrast, ACY3 underwent a spatial redistribution upon neuronal, but not Schwannian, differentiation (Figs. $3A_{C, G, K, O}$). The lack of profound phenotypic alteration in SMS-KCNR cells (Fig. 3B) was not unexpected given that these MYCN-amplified cells were refractory to therapy and likely to remain in an undifferentiated state. Differentiation of SMS-KNCR cells in media containing 1% FBS was not remarkably different (data not shown). However, cAMP treatment of growth arrested SK-N-SH cells (Fig. 4) displayed significant differences to cells differentiated in media containing 10 % FBS (Fig. 3A). Both ACY1 (Figs. 4A, D) and ACY3 (Figs. 4C, F) were robustly expressed in differentiated neurons. Unlike arrested neuroblastic cells, which lacked appreciable ASPA expression (Fig. 4B arrows), cAMP differentiated neuronal cells expressed ASPA (Fig. 4E arrows), particularly at points of cell-cell contact (Fig. 4E arrowheads). Thus, similar to basal expression in neuroblastoma cells (Fig. 1), ASPA expression in differentiated cells is distinct from that of ACY1 and ACY3.

Aminoacylases are differentially expressed in human tissue *in vivo*

Since the majority of neuroblastoma tumors form in a suprarenal location and likely arise from the adrenal gland, immunohistochemical analysis of human biopsy tissue arrays was undertaken to characterize aminoacylase expression in normal human adrenal gland and cancers arising within the adrenal gland. Similar to that observed *in vitro*, aminoacylases were differentially expressed *in vivo* (Fig. 5). While all three aminoacylases were expressed in the juvenile (i.e., 15-year-old female) adrenal medulla (Figs. 5A1–C1), as determined by tyrosine hydroxylase immunoreactivity (Fig. 5D) and morphology (Fig. 5E), ASPA was the most abundantly expressed (Fig. 5B1). In contrast, in the adult (i.e., 40-year-old female) adrenal gland, ACY3 (Fig. 5C2) was weakly expressed in the medulla, while ACY1 (Fig. 5A2) and ASPA (Fig. 5B2) were more abundantly expressed in the adrenal cortex. Similar to their increased expression in MYCN-amplified cell lines *in vitro*, ACY1 (Fig. 5F) and ACY3 (Fig. 5H) expression was detected in neuroblastoma biopsy specimens. In contrast,

ASPA expression was not detectable (Fig. 5G) in the two neuroblastoma specimens examined (Figs. 5F–J1 from a 12-year-old female; Figs. 5F–J2 from a 20-year-old male). To assess whether this expression profile was unique to neuroblastoma, aminoacylase expression was examined in pheochromocytoma (Figs. 5K–O; from a 50-year-old male) and adenocarcinoma (Figs. 5P–T; from a 27-year-old female), cancers of the adrenal medulla and cortex, respectively. Similar to neuroblastoma, ACY1 was robustly expressed in both cancer types (Figs. 5K, P). However, ASPA (Figs. 5L, Q) and ACY3 (Figs. M, R) expression were contrary to that of neuroblastoma (i.e., ASPA abundantly expressed and ACY3 weakly expressed). Given the distinct aminoacylase spatial distribution *in vitro* and *in vivo* and regulation in neuroblastoma cell lines, it suggests each aminoacylases likely subserves different functions.

Discussion

Unfortunately, the identification of genetic correlates of high-risk/poor prognosis neuroblastoma (e.g. BDNF/TrkB expression, MYCN amplification) has not consistently translated into equal gains in treatment efficacy, with long-term overall survival for children with advanced disease remaining less than 40% in spite of increasingly intensive chemotherapy regimens.[26, 27] Therefore, continued examination for additional developmental and molecular alterations contributing to the etiology of neuroblastoma initiation or progression is of paramount clinical importance.

Previous reports have postulated a putative tumor suppressor role for ACY1.[9, 10] Despite this proposed role, ACY1 expression was paradoxically greater in MYCN-amplified SMS-KCNR cells than non-MYCN-amplified SK-N-SH cells. Yet, ACY1 was the only aminoacylase whose expression was markedly up-regulated upon differentiation in media containing 10% serum, supporting a role for ACY1 in differentiation. Conversely, ACY1 expression was decreased in SMS-KCNR cells upon differentiation. Differential subcellular localization is one possible explanation for these incongruent observations. In SK-N-SH cells, ACY1 expression was primarily centriolar, but in SMS-KCNR cells, expression was more diffusely cytoplasmic. ACY1 regulates the subcellular localization of sphingosine kinase type 1 and, thereby, its product sphingosine-1-phosphate, promotes cell growth and inhibits apoptosis of tumor cells.[28] Therefore, the distinct subcellular localization of ACY1 might similarly be involved in targeting specific protein substrates in neuroblastoma cells. Curiously, induction of neural differentiation in SK-N-SH cells altered expression of the 65 kDa, but not the predicted 50 kDa, ACY1 isoform. All three aminoacylases exist in multimeric forms that are enzymatically active deacetylases[29–31] and are quite atypical “cytoplasmic” enzymes in that they may undergo glycosylation and phosphorylation.[12] Glycosylation represents the most plausible explanation for the 15 kDa increase in molecular weight, but future studies will need to be undertaken to confirm this notion. The disparate result of increased ACY1 expression in MYCN-amplified cells, yet increased expression upon differentiation is mimicked *in vivo*. Kaplan-Meier survival data indicate that high ACY1 expression is correlated with better prognosis, as one would predict for a tumor suppressor. However, high expression is also associated with increased mortality initially (Oncogenomics database: http://pob.abcc.ncifcrf.gov/cgi-bin/JK?rm=get_kms;db=nbProg;exprs=log2ctr;threshold=2;limit=100;geneid=4117); thus, the relevance of ACY1 in neuroblastoma is, at present, unclear.

Although ASPA expression was more highly expressed in the non-MYCN-amplified SK-N-SH line than the MYCN-amplified SMS-KCNR line, this is likely due more to cell type than MYCN status. ASPA is primarily expressed in CNS glial cells and may contribute to a less malignant neuroblastoma phenotype by supporting glial cell differentiation. The enrichment of ASPA in SK-N-SH and SH-EP S-type cells is consistent with Kaplan-Meier survival data

that low ASPA expression correlates with an unfavorable prognosis (Oncogenomics database: http://pob.abcc.ncifcrf.gov/cgi-bin/JK?rm=get_kms;db=nbProg;exprs=log2ctr;threshold=2;limit=100;geneid=9710) in that patients with tumors possessing low levels of Schwannian stroma have a less favorable prognosis.[25] Although ASPA is best characterized for its role in myelin lipid synthesis, by use of the acetate liberated upon NAA deacetylation[11], recent evidence suggests a link between ASPA and cell cycle regulation.[32] ASPA knockout mice exhibited increased G1→S cell cycle progression and increased histone acetylation, consistent with previous findings of nuclear ASPA localization.[22, 30] ASPA may also contribute to neuroblastoma tumorigenesis via its suppressive effect on BDNF expression. ASPA-null rats exhibit abnormally elevated BDNF levels that are diminished upon transfection with constitutive ASPA, placing ASPA regulatory hierarchically above BDNF.[33] Based on its punctate membrane-associated expression, it has been suggested that ASPA may subserve a signaling function.[33] Given that BDNF promotes tumor survival, angiogenesis, and drug resistance in TrkB-positive neuroblastoma tumors[4, 5] and the mechanisms of BDNF/TrkB regulation are poorly understood, a possible link between ASPA and BDNF warrants further investigation.

ACY3 is the least understood aminoacylase primarily due to its limited substrate presence in eukaryotic cells (e.g., N-acetylated aromatic amino acids and mercapturic acids).[13, 14] Nonetheless, data are consistent with a role for ACY3 in neuroblastoma promotion: 1) the 80 kDa ACY3 isoform was more robustly expressed in MYCN-amplified cells, 2) ACY3 was expressed in the juvenile and adult adrenal medulla and abundantly expressed in neuroblastoma surgical specimens, and 3) Kaplan-Meier survival data indicates that high ACY3 expression correlates with poor prognosis (Oncogenomics database: http://pob.abcc.ncifcrf.gov/cgi-bin/JK?rm=get_kms;db=nbProgSg;exprs=log2ctr;threshold=2;limit=100;geneid=30370). However, the one inconsistent finding in this study was ACY3 up-regulation upon cAMP-induced neural differentiation of SK-N-SH cells. Yet, unlike ACY1, this increase only occurred in media containing 1% serum. Another perplexing finding is ACY3's nuclear localization. While nuclear ASPA expression has been reported[30], to our knowledge ACY3 has not been previously detected within the nucleus. The ACY3 peptide used as an immunogen shows no homology to ASPA, ACY3 sera does not cross react with recombinant ASPA, and immunolabeling is blocked by peptide pre-incubation; hence, we believe this immunolabeling to be authentic. However, based on our limited understanding of ACY3's normal physiological role, it is difficult to infer the functional importance of ACY3 expression in neuroblastoma.

The primary role of aminoacylases is to deacetylate amino acids for subsequent use in protein synthesis, or, in the case of ASPA, to provide acetate for myelin synthesis. Although the conservation of the essential zinc-binding functional groups and zinc-dependence for enzymatic activity demonstrate that aminoacylases are zinc–dependent metalloproteinases[34], thus far no proteolytic substrates have been identified. Therefore, future studies will need to be undertaken to determine whether the aminoacylases function via their traditional, amino acid deacetylation activity or some, as yet unidentified, novel mechanism and whether their role is restricted to neuroblastoma or is of relevance to other tumors.

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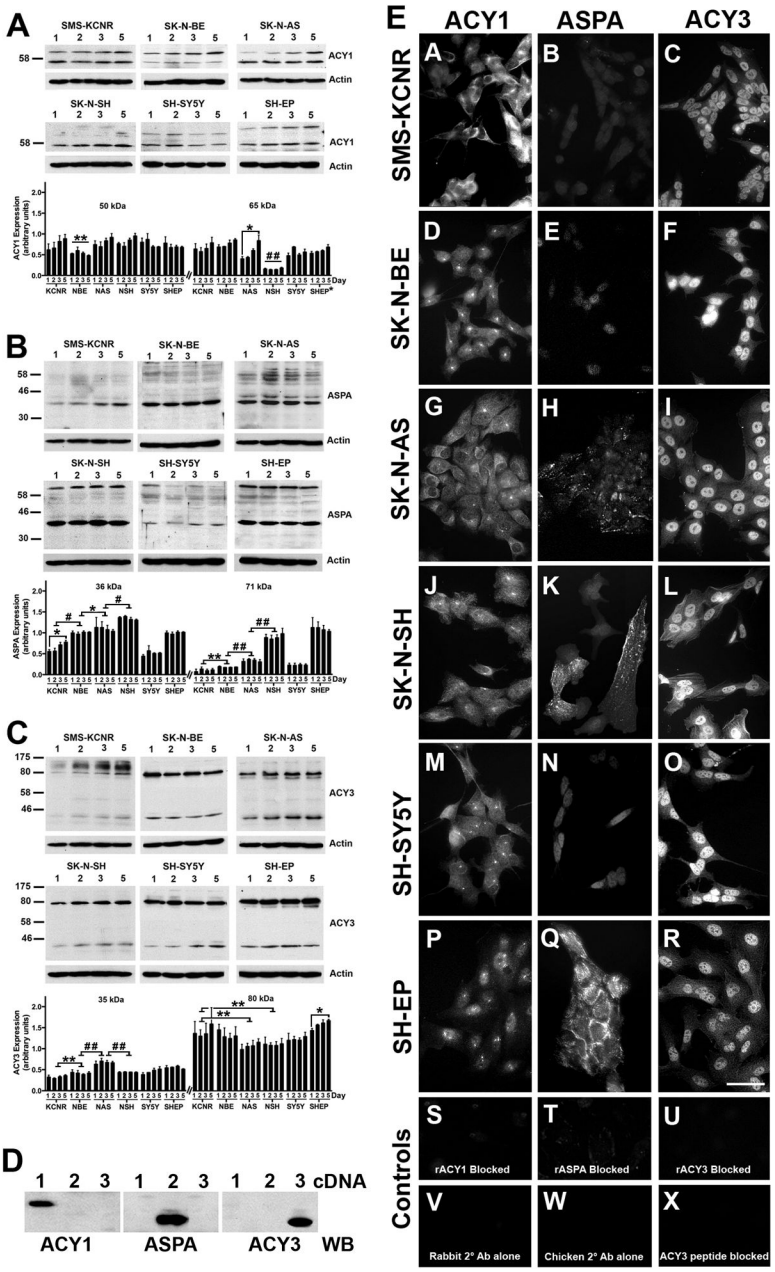

Figure 1. Aminoacylases are differentially regulated in neuroblastoma cell lines

Densitometric analysis of ACY1 (A), ASPA (B), and ACY3 (C) western blots (25 μg protein normalized to actin). ACY1 was more strongly expressed in MYCN-amplified cell lines ($p = 0.011$), while ASPA expression was more highly expressed in lines with a glial component (i.e., SK-N-SH and SH-EP) ($p < 0.0001$). ACY3 expression was reduced (predicted 35 kDa monomer; $p < 0.001$) or increased (80 kDa dimer; $p = 0.003$) in MYCN-amplified lines. Data are presented as mean ± SEM. n = 3. *$p < 0.05$, **$p \leq 0.01$, ##$p \leq 0.0001$. D) Antibody specificity was demonstrated by western blot analysis of whole cell lysates from cells stably transfected with ACY1, 2, or 3 cDNA. E) Immunocytochemistry after 3 days *in vitro* revealed a distinct subcellular localization for each aminoacylase – cytosolic ACY1, membranous ASPA staining in Schwannian cells, and nuclear ACY3.

Controls include pre-incubation with recombinant aminoacylase protein (S–U) or immunizing peptide (X) or exclusion of primary antibody (V, W). Scale bar = 50 μm.

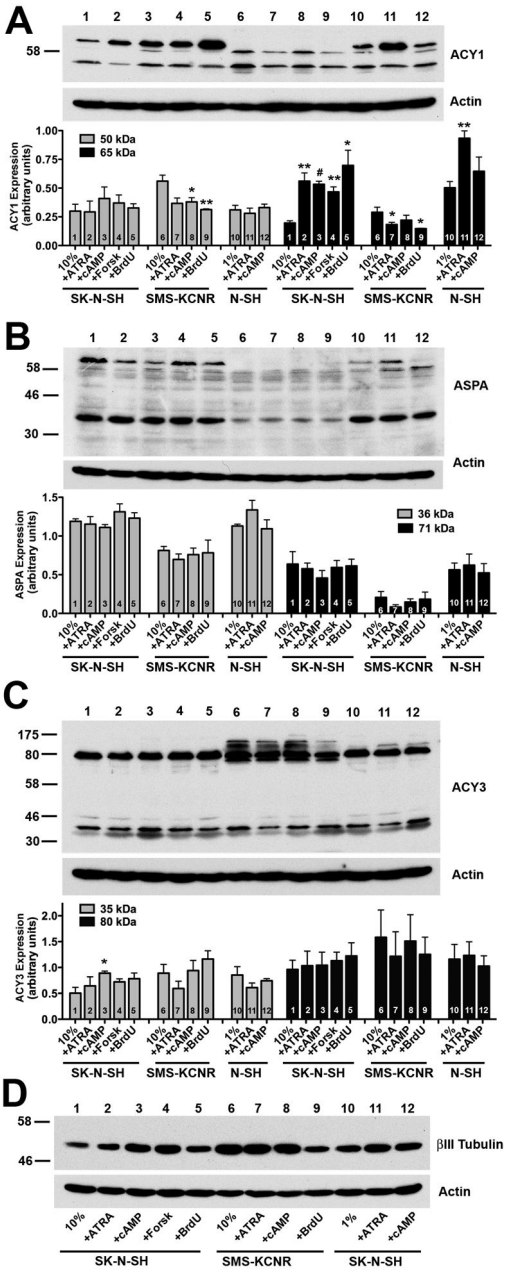

Figure 2. ACY1 expression is differentially regulated in SK-N-SH and SMS-KCNR cells upon differentiation

Densitometric analysis of ACY1 (A), ASPA (B), and ACY3 (C) western blots (25 μg protein normalized to actin) 7 days after differentiation induction. SK-N-SH cells were grown in media containing 10% FBS (1) or with ATRA (2), cAMP (3), forskolin (4), or BrdU (5). Similarly, SMS-KCNR cells were grown in media containing 10% FBS (6) or with ATRA (7), cAMP (8), or BrdU (9). SK-N-SH cells were growth arrested in media containing 1% FBS for 12 hours, then grown in the absence (10), or presence of ATRA (11), or cAMP (12). Only ACY1 was responsive to differentiation factors; expression increased in SK-N-SH cells and decreased in SMS-KCNR cells. Induction of a neuronal phenotype was confirmed by increased βIII-tubulin expression (D). Data are presented as mean ± SEM. n = 3. *$p < 0.05$, **$p \leq 0.01$, #$p \leq 0.001$.

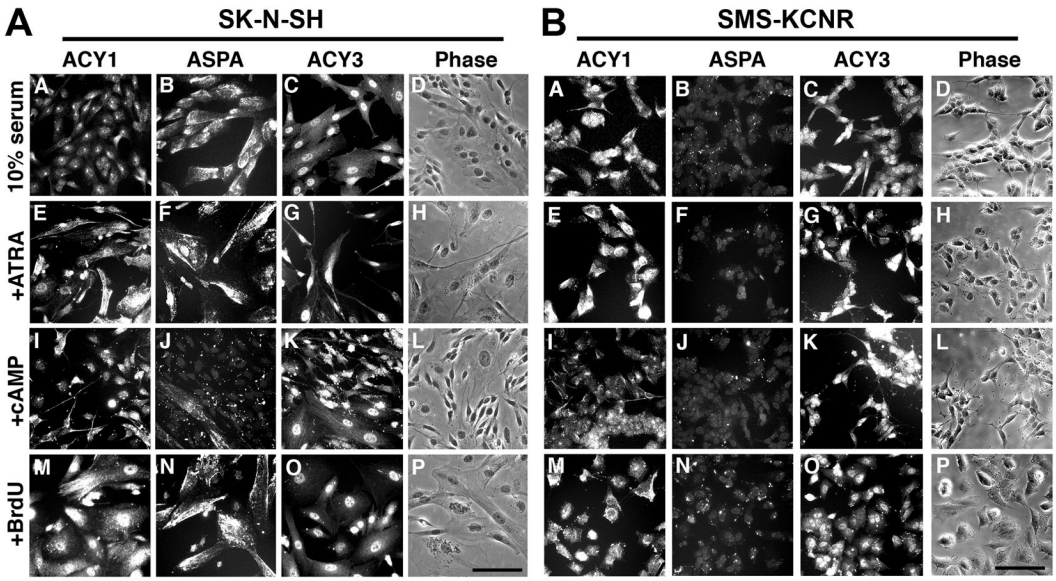

Figure 3. Aminoacylase expression was minimally altered when differentiated in medium containing 10% serum

SK-N-SH (A) and SMS-KCNR (B) cells were maintained in growth media containing 10% FBS in the absence or presence of differentiating agents for 7 days and examined immunocytochemically. Centriolar ACY1 expression (A_A) was lost under neuronal ($A_{E, I}$) and Schwannian (A_M) differentiation conditions. ASPA expression was unaltered, with the exception of decreased expression in the presence of cAMP (A_J). ACY3 expression was more dramatically altered under neuronal ($A_{G, K}$) than Schwannian (A_O) differentiation conditions. Phase-contrast images revealed changes toward neuronal (i.e. process outgrowth) and Schwannian (i.e., larger cell size) cellular morphology. SMS-KCNR cells (B) failed to exhibit profound morphological alterations upon differentiation. Scale bar = 100 μm.

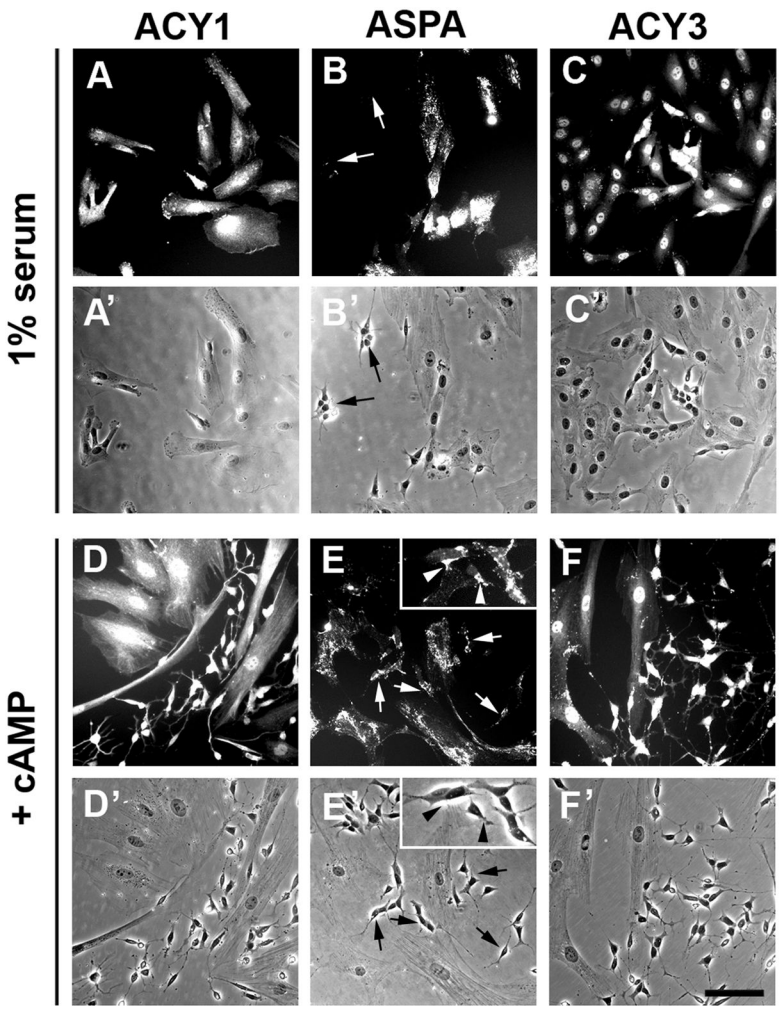

Figure 4. Aminoacylase expression is up-regulated upon neuronal differentiation of SK-N-SH cells

SK-N-SH cells were arrested by growth in media containing 1% FBS for 12 hours prior to neuronal induction with cAMP. Cells were grown in the absence (A–C) or presence of cAMP (D–F) for 7 days and examined immunocytochemically. Expression of both ACY1 (A, D) and ACY3 (C, F) were robustly expressed in differentiated neurons, as revealed by prominent neurite outgrowth (D′, F′). ASPA was expressed in cells with Schwannian morphology (B, B′), but not in arrested cells with neurite-like processes (B, B′ arrows). In contrast, ASPA was expressed in neurons (E, E′ arrows), particularly at points of cell-cell contact (E, E′ arrowheads), in response to cAMP treatment. Scale bar = 100 μm, inset = 50 μm.

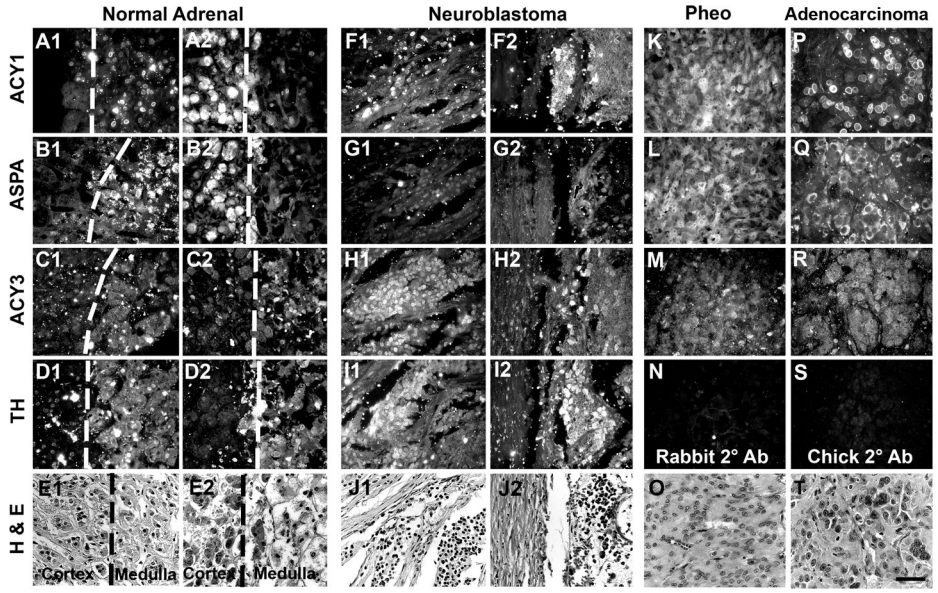

Figure 5. Aminoacylase expression in human adrenal gland and adrenal-derived cancers

Immunohistochemical analysis of aminoacylase expression in human biopsy tissue arrays of normal adrenal (1:15-year-old female, 2:40-year-old female), neuroblastoma (1:12-year-old female, 2:20-year-old male), pheochromocytoma (50-year-old male), and cortical adenocarcinoma (27-year-old female) revealed distinct aminoacylase expression profiles. While ACY1 (A) and ASPA (B) displayed similar expression in normal adrenal, ASPA expression was not detectable in the two neuroblastoma specimens examined (G). In contrast, ACY1 (F) and ACY3 (H) were expressed in neuroblastoma coincident with tyrosine hydroxylase (TH) (I). This expression pattern was unique for neuroblastoma, in that ASPA was expressed in pheochromocytoma (L) and adenocarcinoma (Q), while ACY3 was not abundantly expressed in these cancers (M, R). Scale bar = 50 μm.