N-Acetylaspartate (NAA) and N-Acetylaspartylglutamate (NAAG) Promote Growth and Inhibit Differentiation of Glioma Stem-like Cells*

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Background: N-acetylaspartate (NAA), the primary source of brain acetate, and aspartoacylase (ASPA), the enzyme that catabolizes NAA, are decreased in glioma, thereby decreasing acetate bioavailability.

Results: Treatment with NAA and NAAG promotes growth and inhibits differentiation of glioma stem-like cells (GSCs).

Conclusion: This study links acetate bioavailability and GSC malignancy.

Significance: Because NAA/NAAG-mediated acetate supplementation promoted GSC growth, an alternative acetate therapeutic is required.

Metabolic reprogramming is a pathological feature of cancer and a driver of tumor cell transformation. N-Acetylaspartate (NAA) is one of the most abundant amino acid derivatives in the brain and serves as a source of metabolic acetate for oligodendrocyte myelination and protein/histone acetylation or a precursor for the synthesis of the neurotransmitter N-acetylaspartylglutamate (NAAG). NAA and NAAG as well as aspartoacylase (ASPA), the enzyme responsible for NAA degradation, are significantly reduced in glioma tumors, suggesting a possible role for decreased acetate metabolism in tumorigenesis. This study sought to examine the effects of NAA and NAAG on primary tumor-derived glioma stem-like cells (GSCs) from oligodendrogliaoma as well as proneural and mesenchymal glioblastoma, relative to oligodendrocyte progenitor cells (Oli-Neu). Although the NAA dicarboxylate transporter NaDC3 is primarily thought to be expressed by astrocytes, all cell lines expressed NaDC3 and, thus, are capable of NAA up-take. Treatment with NAA or NAAG significantly increased GSC growth and suppressed differentiation of Oli-Neu cells and proneural GSCs. Interestingly, ASPA was expressed in both the cytosol and nuclei of GSCs and exhibited greatest nuclear immunoreactivity in differentiation-resistant GSCs. Both NAA and NAAG elicited the expression of a novel immunoreactive ASPA species in select GSC nuclei, suggesting differential ASPA regulation in response to these metabolites. Therefore, this study highlights a potential role for nuclear ASPA expression in GSC malignancy and suggests that the use of NAA or NAAG is not an appropriate therapeutic approach to increase acetate bioavailability in glioma. Thus, an alternative acetate source is required.

Glioma, the most common adult primary brain cancer, is lethal due to a near inevitable post-surgical tumor recurrence due, in part, to radiation- and chemotherapy-resistant glioma stem-like cells (GSCs). Because oligodendrocyte progenitor cells (OPCs) are a glioma cell of origin (1), an understanding of the mechanisms regulating OPC proliferation and differentiation is important toward developing effective therapies against GSCs.

N-Acetylaspartate (NAA) is the second most abundant amino acid derivative in the brain, second only to glutamate, and is the most abundant source of acetate (2). NAA is synthesized in neuronal mitochondria (3) or endoplasmic reticulum (4) via acetylation of aspartate by the enzyme aspartate N-acetyltransferase (Fig. 1). NAA is the primary storage form of metabolic acetate needed for postnatal myelin lipid synthesis but is also hypothesized to contribute to neuronal mitochondrial metabolism, neuronal osmoregulation, and axon-glial signaling (5). Additionally, NAA may be converted to the dipeptide neurotransmitter N-acetylaspartylglutamate (NAAG) by the enzymes NAAG synthetase I and II (6, 7). NAAG selectively activates metabotropic glutamate receptor type 3 (GRM3) and is restored to NAA by glutamate carboxypeptidases (GCPII/III) expressed on the outer surface of astrocytes (8). Accordingly, NAAG serves as a source of persynaptic NAA and glutamate.

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2 The abbreviations used are: GSC, glioma stem-like cell; OPC, oligodendrocyte progenitor cell; AceCS1, acetyl-CoA synthase 1; ASPA, aspartoacylase; CD, Canavan disease; CNPase, 2',3'-cyclic nucleotide 3'-phosphodiesterase; DM, differentiation medium; GBM, glioblastoma multiforme; GCPl/II, glutamate carboxypeptidase II and III; GPAP, glial fibrillary acidic protein; GRM, metabotropic glutamate receptor; IDH, isocitrate dehydrogenase; NAA, N-acetylaspartate; NAAG, N-acetylaspartylglutamate; NaDC3, sodium-dependent dicarboxylate transporter 3; PLL, poly-L-lysine; SCM, stem cell medium; PN, proneural; MES, mesenchymal.
Although NAA is of neuronal origin, its primary site of catabolism is in oligodendrocytes where it is hydrolyzed by the enzyme aspartoacylase (ASPA) to generate free acetate and aspartate (9). NAA-derived acetate needs to be converted to acetyl-coenzyme A (acetyl-CoA) via the cytosolic/nuclear enzyme acetyl-CoA synthetase 1 (AceCS1) for lipid biosynthesis and histone/protein acetylation (10) or mitochondrial acetyl-CoA synthetase 2 for use in the Krebs cycle (11). Liberated aspartate may generate oxaloacetate for utilization in the Krebs cycle or may be used for protein synthesis (5). Although best characterized as a cytosolic lipogenic enzyme (12), ASPA may also serve as an acetate source for the acetylation of protein substrates especially in oligodendrocytes where ASPA is abundant. The nuclear co-localization of ASPA (13) and AceCS1 (14) suggests that ASPA-mediated NAA catalysis may provide acetate for histone acetylation, thereby regulating gene expression necessary for oligodendrocyte differentiation. Thus, ASPA and AceCS1 possess functions distinct from their traditional roles in myelination (15).

Levels of NAA and NAAG are dysregulated in various disease states associated with aberrant oligodendrocyte differentiation. Missense mutations in ASPA result in Canavan disease (CD), a fatal childhood leukodystrophy characterized by white matter dysmyelination and spongiform degeneration arising from the inability to hydrolyze NAA and liberate acetate for myelin lipid synthesis (16). Rodent models of CD display defects in oligodendrocyte maturation and increased OPC proliferation, providing a possible link between NAA catabolism and OPC cell cycle arrest and/or oligodendrocyte differentiation (17, 18). Whereas increased NAA levels exemplify CD, levels of both NAA and NAAG are decreased in glioma tumors (19, 20) coincident with decreased ASPA expression.3 Inasmuch as ASPA deficiency is associated with increased OPC proliferation in CD, ASPA is decreased in glioma, and OPCs are a cellular source for gliomagenesis, ASPA and NAA/NAAG may be possible targets for therapeutic intervention. This study sought to determine the effects of NAA and NAAG supplementation on OPC and GSC proliferation and differentiation. Collectively, our results show that NAA and NAAG promote GSC growth and inhibit GSC differentiation. Therefore, metabolic changes that increase the abundance of NAA or NAAG may exacerbate glia tumor pathology by reinforcing a proliferative undifferentiated GSC phenotype.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Oli-Neu cells, derived from mouse OPCs and immortalized by stable constitutive expression of the ErbB2 receptor (21), were grown on poly-L-lysine (PLL; 10 μg/ml)-coated dishes in SATO growth medium (DMEM with 0.1 mg/ml apotransferrin, 0.01 mg/ml insulin, 400 nm triiodothyronine, 2 mM glutamine, 220 nm sodium selenite, 500 nm thyr oxine, 1% horse serum, and 25 μg/ml G418) (22). Human cerebral cortical astrocytes (HA#1800 ScienCell; Carlsbad, CA) were cultured in basal medium with 2% fetal bovine serum and astrocyte growth supplement (AM#1801 ScienCell). GSCs were maintained in DMEM/F-12 (Corning; Tewksbury MA) supplemented with 2 μM glutamine, 1× B27 (Invitrogen), 20 ng/ml EGF, and 20 ng/ml basic FGF (PeproTech; Rocky Hill, NJ). All media contained 50 units/ml penicillin and 50 μg/ml streptomycin (Invitrogen). For pharmacological treatments, cells (20,000 cells per well of a 24-well plate or 10,000 cells per cm² per 6-cm dish) were cultured overnight in growth medium then treated with NAA (100 μM) or NAAG (10 μM) (Sigma). Medium was replenished every 48 h, and cells were harvested after 2, 4, and 6 days in vitro (i.e. 1, 3, 5 days of treatment).

Oli-Neu differentiation was induced using modified SATO medium (DMEM supplemented with 100 μg/ml apotransferrin, 5 μg/ml insulin, 60 nm triiodothyronine, 30 nm sodium selenite, 100 μM putrescine, 1% horse serum, and 25 μg/ml G418) (23) upon plating with the addition of dibutyryl-cAMP (1 μM, Sigma) for up to 5 days or with the ErbB2 antagonist PD174265 (1 μM, sc-204170, Santa Cruz Biotechnology; Santa Cruz, CA) (24) for 48 h. GSC differentiation was induced in differentiation media (DM; DMEM with 10% fetal bovine serum). Medium was replenished every 48 h.

Growth dynamics were assessed using unbiased trypan blue exclusion-based cytometry. Cells were plated (at 10,000 cells per well of a 24-well plate) directly in the absence or presence of

3 A. R. Tsen and D. M. Jaworski, submitted for publication.
NAA (100 μM), NAAG (10 μM), or glutamate (10 μM, 50 nM). After 1, 3, and 5 days of treatment, cells were counted according to the manufacturer’s instructions (Countess Automated Cell Counter; Invitrogen).

**mRNA Expression Analysis**—GSCs were cultured as non-adherent spheres in stem cell medium (SCM), and Oli-Neu cells were cultured on PLL in SATO media at a density of 2 × 10^5 cells/well of a 6-well plate. After 4 days, total RNA was isolated using STAT-60 (TelTest Inc.; Friendswood, TX), and DNase was treated using the SV Total RNA isolation system (Promega; Madison, WI). RNA (2 μg) was reverse-transcribed using SuperScript II reverse transcriptase and random hexamers (Invitrogen). Adult mouse cerebral cortex, human anaplastic oligodendroglioma, and glioblastoma tumors served as positive controls (Promega). Adult mouse cerebral cortex, human anaplastic oligodendroglioma, and glioblastoma tumors served as positive controls (Promega; Madison, WI). RNA (2 μg) was reverse-transcribed using SuperScript II reverse transcriptase and random hexamers (Invitrogen).

**Western Blot Analysis**—To determine ASPA spatial localization, cells (2.5 × 10^4 cells/10 cm dish) were cultured in DM for 4 days, collected by trypsinization (0.025% trypsin/EDTA), centrifuged at 1500 rpm for 5 min, and then washed with Dulbecco’s phosphate-buffered saline. Cells (1 × 10^6) were resuspended in 200 μl of buffer A (10 mM Heps, pH 7.6, 10 mM KCl, 0.1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine) with protease inhibitors (2 μM dithiothreitol, 2.5 μM phenylmethylsulfonyl fluoride, 100 μM Na_2MoO_4, and 5 μg/ml aprotinin, leupeptin, and pepstatin) and incubated on ice for 15 min. Nonidet P-40 (IgepalCA-630, 12.5 μl of 10%) was added dropwise while vortexing for 10 s. After centrifugation at 1300 rpm for 30 s, the supernatant (cytosolic fraction) was removed and stored at −80 °C. Radiimmunoprecipitation assay buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) plus protease inhibitors (25 μl) were added to the remaining pellet, and samples were heated for 5 min at 95 °C and pulse-sonicated 10 times for 1 s each. These samples were then stored at −80 °C as the nuclear fraction.

**Western Blot Analysis**—SDS-PAGE (25 μg of protein from whole cell lysates), and Western blotting were performed as previously described (26). For antibody blocking, ASPA antibody (0.4 μg, GTX13389 GeneTex) was incubated with either a 5-fold (blocking of novel ASPA species) or 10-fold (blocking of 36-kDa ASPA protein) molar excess of ASPA protein (GTX110699-PRO GeneTex) in Tris-buffered saline (10 mM Tris, pH 7.4, 150 mM NaCl) with 0.4% Tween 20 and 3% bovine serum albumin. To increase protein/antibody interaction, samples were incubated at 37 °C for 1 h (novel ASPA species) or 2 h (36 kDa ASPA) before overnight incubation at 4 °C with shaking. Immune complexes were centrifuged for 15 min at 1300 rpm at 4°C, and the ASA-depleted supernatant was incubated with Western blots containing 10 μg of protein (whole cell lysate). Immunocomplexes were visualized by enhanced chemiluminescence (PerkinElmer Life Sciences), and densitometry was performed using Quantity One software (Bio-Rad).

**Fluorescence Microscopy**—Immunocytochemistry was performed as previously described (27). For antibody blocking, ASPA antibody (0.3 μg) was incubated in a 10-fold molar excess of ASPA protein in blocking buffer (DMEM with 5% FBS, 0.1% glycine, 0.1% l-lysine, 0.2% sodium azide). Samples were incubated at 37 °C for 1 h and processed as described above for Western blot analysis. Immunoreactivity was visualized with a Nikon epifluorescence microscope (MicroVideo Instruments;
RESULTS

NAA and NAAG Differentially Regulate ASPA Expression and OPC Differentiation—NAA levels and ASPA expression are up-regulated during oligodendrocyte differentiation (18, 28), and ASPA-mediated NAA catabolism is necessary for proper oligodendrocyte maturation in vivo (17, 18). Conversely, NAAG is proposed to promote survival and expansion of neural progenitor cells in vitro through GRM3 activation (29). However, the effects of NAA and NAAG on OPCs have not been directly investigated. Therefore, the influence of physiological levels of NAA or NAAG (100 and 10 μM, respectively) on ASPA expression and differentiation were examined using a murine OPC line (Oli-Neu) that differentiates to oligodendrocytes with high fidelity. Neither NAA nor NAAG altered Oli-Neu growth (Fig. 2A). Oli-Neu cells expressed the NAA transporter NaDC3 (Fig. 2B); thus, they are competent to up-take NAA. To our knowledge this is the first report of NaDC3 expression in cells of an oligodendroglial lineage. NAAG may be hydrolyzed to NAA and glutamate by GCPII/III or function as a group II metabotropic glutamate receptor agonist, with high selectivity for GRM3 over GRM2 (8). GRM5, a group I metabotropic glutamate receptor, is expressed by neural progenitor cells and OPCs and is implicated in progenitor cell proliferation and survival (30, 31). Although NAAG does not stimulate GRM5 (8), its expression was nonetheless assessed. GRM5, but not GRM3 or GRM2, mRNA was detected in Oli-Neu cells (Fig. 2B). Because Oli-Neu cells did not express detectable GCPII/III mRNA, NAAG may remain as an intact dipeptide and function in the absence of GRM3 or be hydrolyzed to NAA and glutamate via a GCPII/III-independent mechanism. In the CNS, GCPII expression is primarily restricted to astrocytes (32); thus, its absence in Oli-Neu cells is consistent with their OPC phenotype.

Oli-Neu cells demonstrated a temporal increase in ASPA expression with time in culture (Fig. 2C). This temporal increase in ASPA expression is likely not due to spontaneous differentiation induced in confluent cultures as no change in CNPase expression was detected (Fig. 2C). Interestingly, ASPA was expressed as a 36–38-kDa immunoreactive doublet. Whether this doublet reflects post-translational modifications (e.g. phosphorylation, glycosylation) (33) is not known. Although NAA transiently increased ASPA expression, this was not associated with a concordant increase in CNPase expression (Fig. 2C) but was associated with increased process outgrowth (Fig. 2D). In contrast, NAAG treatment abrogated increased ASPA expression (Fig. 2C) and reduced CNPase expression (Fig. 2C) and process outgrowth (Fig. 2D). These results reveal an inhibitory role for NAAG on oligodendrocyte differentiation of Oli-Neu cells.

NAA Inhibits cAMP-induced Oli-Neu Cell Differentiation—To determine whether increased ASPA expression failed to promote Oli-Neu CNPase expression and differentiation due to its inability to overcome constitutive ErbB2 expression, cells were induced to differentiate using cAMP (21) or inhibition of ErbB2 signaling using the ErbB2 antagonist PD174265 (24) (Fig. 2E–G). Treatment with cAMP induced morphological alterations reflective of differentiated oligodendrocytes (e.g. highly arborized processes) (Fig. 2E) and increased CNPase expression; however, cAMP significantly reduced ASPA expression (Fig. 2F). Although NAA alone did not alter ASPA or CNPase expression, NAA abolished the cAMP-induced reduction of ASPA expression and attenuated the cAMP-mediated increase in CNPase (Fig. 2F), with the majority of cells retaining their undifferentiated bipolar morphology (Fig. 2E). Differentiation induced by ErbB2 inhibition did not alter ASPA expression but induced more robust morphological alterations and increased CNPase expression more profoundly than cAMP (Fig. 2G). Collectively, these data support a cAMP-mediated regulation of ASPA rather than a differentiation-mediated regulation. Furthermore, in the context of cAMP signaling, NAAG appears to antagonize OPC differentiation.

NAA and NAAG Promote Growth of Oligodendroglioma Stem-like Cells—Because mouse models of oligodendroglioma reveal transformed cells with phenotypic qualities similar to OPCs (34) and NAA and NAAG are decreased in glioma (19, 20), the effects of NAA or NAAG on cell growth were assessed in GSCs derived from oligodendroglioma tumors. NAA and NAAG exerted a growth-promoting effect on the more malignant anaplastic OG35 GSCs but not the grade II OG33 GSCs (Fig. 3A). Although some commercial preparations of NAAG have been reported to contain contaminating glutamate (between 0.1 and 0.5%) (8), neither 50 nM glutamate (equivalent to 0.5% contamination of 10 μM NAAG) nor 10 μM glutamate promoted OG35 GSC growth (Fig. 3B). Similar to Oli-Neu cells, OG33 and OG35 GSCs expressed NaDC3 but not GCPII/III (Fig. 3C) and, thus, are capable of NAA up-take but not GCPII/III-mediated NAAG catabolism. Unlike the murine GRM3 gene, which encodes a single mRNA transcript, the human GRM3 gene codes for multiple mRNA splice variants. GRM3 expression was not detectable in OG GSCs using primers spanning exons 5–6 (Fig. 3C) and exons 2–3 (data not shown). Even using primers that would detect all variants, GRM3 mRNA was not detected in OG33 and OG35 GSCs (data not shown). Although the absence of GCPII/III suggests that NAAG promotes growth by signaling as an intact dipeptide, neither its cognate receptor GRM3 nor the related GRM2 and GRM5 receptors were detected in OG35 GSCs (Fig. 3C). Additionally, the more pronounced growth-enhancing effect of NAAG over NAA occurs independently of glutamate contamination or from glutamate derived by NAAG degradation.

NAA and NAAG Induce the Expression of a Novel 26-kDa Immunoreactive ASPA Species in Oligodendroglioma GSC Nuclei—inasmuch as NAA levels are significantly reduced in glioma tumors relative to normal brain tissue (19), a parsimonious explanation for decreased ASPA expression in glioma in...
vivo is a compensatory negative feedback mechanism due to reduced bioavailability of NAA, the only known substrate of ASPA (35). Thus, the regulation of ASPA expression was examined in GSCs grown in stem cell medium (SCM) and when induced to differentiate in DM in the absence and presence of NAA and NAAG. ASPA was expressed at comparable levels in SCM and DM (Fig. 3D). NAA and NAAG treatment induced a modest increase in ASPA expression in OG33 GSCs, whereas only NAAG increased ASPA expression in OG35 GSCs (Fig. 3D). Curiously, NAA and NAAG induced the expression of a novel ~26-kDa immunoreactive ASPA species exclusively in OG33 GSCs (Fig. 3D). Consistent with previous reports that ASPA is a cytosolic-nuclear protein (13, 14), subcellular fractionation of GSCs grown in DM with NAAG revealed the putative 36-kDa ASPA in both the cytosol and nucleus (Fig. 3E). Strikingly, the ~26-kDa ASPA protein was expressed exclusively in OG33 nuclei (Fig. 3E). Among the various ASPA mutations detected in CD, a single base change at Tyr-231 occurs
NAA and NAAG Inhibit Glioma Stem-like Cell Differentiation

NAA and NAAG Inhibit Glial, but Not Neural, Differentiation in GBM GSCs—We next sought to determine if NAA and NAAG regulated growth or differentiation of proneural GBM GSCs. NAA, but not NAAG, promoted proneural GBM GSC growth in SCM (Fig. 5A). Serum-induced differentiation was associated with reduced growth, which was unaltered by NAA or NAAG (Fig. 5B). The proneural GBM GSCs expressed much lower levels of NaDC3 (Fig. 5C) than Oli-Neu cells (Fig. 2B) or OG GSCs (Fig. 3C). Unlike oligodendroglial GSCs, proneural GBM GSCs expressed high levels of GCPII/III; hence, NAAG can be hydrolyzed to NAA and glutamate (Fig. 5C). Growth factor depletion of the proneural GBM GSCs was associated with significantly increased expression of the astrocytic marker GFAP and the neuronal marker Tuj1, but there was no change in CNPase expression (Fig. 5D). NAA and NAAG reduced glial differentiation capacity (i.e. attenuated GFAP expression, reduced basal levels of CNPase) without a corresponding increase in neuronal cell fate (i.e. unaltered Tuj1 expression) (Fig. 5D). Collectively, these results suggest that NAA and NAAG suppress the expression of genes associated with glial, but not neuronal, differentiation.

ASPA Is Differentially Expressed in Proneural and Mesenchymal GBM GSCs—Unlike oligodendroglial GSCs, proneural GBM GSC differentiation was associated with increased ASPA expression, which, similar to Oli-Neu cells, was present as a 36–38-kDa immunoreactive doublet (Fig. 5E). Treatment with NAAG, but not NAA, attenuated increased ASPA expression (Fig. 5E). Immunocytochemical analysis revealed diffuse cytoplasmic and intense nuclear ASPA staining in GSCs (Fig. 5F). Upon growth factor withdrawal, the cells differentiated and retained cytoplasmic and nuclear ASPA expression, which was largely unchanged by NAA or NAAG.

Inasmuch as mesenchymal GBM GSCs exhibit a more glycolytic metabolism (38), the effect of NAA and NAAG on cell growth was examined in mesenchymal GBM GSCs (Fig. 6). Unlike proneural GBM GSCs, which significantly up-regulated GFAP expression in DM, the mesenchymal GBM GSCs did not express GFAP in SCM or DM (Fig. 6A). Conversely, the mesenchymal GBM GSCs expressed abundant CD44 in SCM, which decreased upon differentiation. The proneural GBM GSCs lacked CD44 in SCM but significantly increased expression when induced to differentiate so that CD44 can support process outgrowth. Upon differentiation of proneural GBM GSCs, ASPA was diffusely expressed throughout the cytoplasm where it was co-expressed with GFAP, nestin (Fig. 6B), and Tuj1 (not shown). In contrast, in the mesenchymal GBM GSCs, ASPA expression was enriched within the nucleus, and these cells lacked GFAP, nestin (Fig. 6B), and Tuj1 (not shown) expression but were Ki67-positive and, thus, maintained their proliferative capacity despite growth in differentiation permissive conditions. Because mesenchymal gliomas are associated with a poor prognosis (39), it was not surprising that growth of the mesenchymal GBM GSCs was greater than the proneural GBM GSCs in SCM (Fig. 6C). Furthermore, because the mesenchymal GBM GSCs do not differentiate (Fig. 6B), their proliferation in DM was even greater than in SCM. The mesenchymal GBM GSCs expressed NaDC3, GRM2, and GRM 5 but no GCPII/III or GRM3 (Fig. 6D), similar to the OG GSCs (Fig. 3C). However, NAA and NAAG increased OGG5 growth, whereas neither affected growth of the mesenchymal GBM GSCs (Fig. 6C). Thus, although the mesenchymal GBM GSCs share phenotypic similarities with OG33 and OG35 GSCs (i.e. nuclear proliferation in DM was even greater than in SCM. The mesenchymal GBM GSCs expressed NaDC3, GRM2, and GRM 5 but no GCPII/III or GRM3 (Fig. 6D), similar to the OG GSCs (Fig. 3C). However, NAA and NAAG increased OGG5 growth, whereas neither affected growth of the mesenchymal GBM GSCs (Fig. 6C). Thus, although the mesenchymal GBM GSCs share phenotypic similarities with OG33 and OG35 GSCs (i.e. nuclear

FIGURE 2. NAA and NAAG regulate ASPA expression and inhibit Oli-Neu differentiation. A, NAA (100 μM) and NAAG (10 μM) had no effect on Oli-Neu cell growth in growth medium (GM). B, Oli-Neu cells express the NaDC3 NAA transporter and, thus, are capable of NAA uptake. Because Oli-Neu cells lack GCP to catalyze NAAG to NAA and glutamate, as an intact dipetide or be cleaved by alternative carboxypeptidases with NAAG hydrolytic activity. If NAAG suberves a signaling function, it does so independent of GRM3 as the mRNA of this receptor was not detected. Mouse cortex (ms CTX) served as a positive control, and actin served as a loading control. C, Oli-Neu cells were evaluated for changes in ASPA and CNPase expression in response to NAA or NAAG. NAA accelerated, whereas NAAG blunted the temporal increase of ASPA expression. NAAG also decreased CNPase expression, a.u., arbitrary units. D, immunocytochemistry of ASPA and CNPase after 4 days in vitro revealed diffuse somatic labeling that was unaltered by NAA or NAAG. E, Oli-Neu cells adopted a mesenchymal tumor phenotype and may not be an ideal model to investigate the role of NAA, NAAG, and ASPA in glial differentiation.
ASPA expression and limited differentiation potential), NAA and NAAG exerted different growth effects.

Finally, to assess its role in differentiation, nuclear ASPA expression was compared in the proneural and mesenchymal GBM GSCs. Total ASPA expression did not differ in proneural and mesenchymal GSCs but they expressed distinct ASPA isoforms (Fig. 6E). When cultured in DM, the proneural GBM GSCs increased expression of the higher molecular weight NAA and NAAG Inhibit Glioma Stem-like Cell Differentiation
component of the ASPA doublet (−38 kDa). In the mesenchymal GBM GSCs, a novel ~24-kDa immunoreactive ASPA isoform was observed. Interestingly, unlike OG33 GSCs, in which the novel ASPA isoform was induced by NAA or NAAG (Fig. 3D), in the mesenchymal GBM GSCs, this isoform was constitutively expressed. Peptide blocking experiments resulted in a loss of reactivity of the 38- and 24-kDa immunoreactive isoforms and diminished reactivity of the 36-kDa ASPA (Fig. 6F). Blocking was also more efficient for ASPA immunolabeling of proneural GBM GSCs (Fig. 6G). Subcellular fractionation revealed that ASPA primarily partitioned to the cytosolic fraction in proneural GBM GSCs (Fig. 6G). In contrast, in the mesenchymal GBM GSCs, full-length ASPA (−36 kDa) was expressed exclusively in the cytosol, whereas the novel ~24-kDa ASPA isoform partitioned exclusively to the nuclear fraction. NAA or NAAG did not discernibly alter the subcellular distribution of ASPA (not shown). Collectively, these results identify a unique pattern of ASPA expression that corresponds with increased GSC proliferation and altered differentiation potential.

**DISCUSSION**

Although diminished NAA magnetic resonance spectroscopy signal is a well recognized feature of glioma tumors, the
physiological consequences of reduced NAA or NAAG bioavailability are a poorly understood area of glioma pathology. This study sought to determine the effects of physiological NAA and NAAG supplementation on OPC and GSC proliferation and differentiation. Our results show that these metabolites promote GSC growth and attenuate glial differentiation, suggesting that increased NAA and NAAG may lead to dysregulated OPC differentiation and exacerbate glioma progression.

**FIGURE 5. NAA and NAAG promote growth and inhibit glial differentiation of proneural GBM GSCs.** A and B, NAA (100 μM), but not NAAG (10 μM), promoted PN GBM GSC growth when cultured in SCM (A), but neither metabolite altered growth when cultured in DM (B). Increased growth was not due to increased survival as no difference in activated caspase-3 was observed (not shown), and cell viability was not affected by time in culture (p = 0.73) or treatment (p = 0.54). At 5 days, % viable cells: 95.0 ± 2.0% SCM, 95.5 ± 0.58% NAA, 95.3 ± 0.44% NAAG. C, PN GBM-derived GSCs express less NaDC3 NAA transporter than Oli-Neu cells (Fig. 2B) and oligodendroglioma GSCs (Fig. 3C). Also, PN GBM GSCs are unique in the expression of GCP; thus, NAAG may be cleaved to generate extracellular NAA and glutamate. Anaplastic oligodendroglioma (Oligo) and GBM tumors served as positive controls, and GAPDH served as a loading control. D, NAA and NAAG inhibited expression of glial (CNPase and/or GFAP) but not neuronal (Tuj1) differentiation markers. SPH, cells grown as free-floating spheres. E, ASPA expression was up-regulated in PN GBM GSCs coincident with differentiation in DM. ASPA expression was attenuated by NAAG. F, after 4 days in SCM (adherent on PLL), ASPA was abundantly expressed in the cytosol and nucleus of PN GSCs. In DM, ASPA was predominantly cytosolic in cells that adopted an elongated differentiated morphology (see Fig. 6B). a.u., arbitrary units. Molecular masses: ASPA = 36 kDa; CNPase = 46–48 kDa; GAPDH = 36 kDa; GFAP = 50 kDa; TUJ1 = 55 kDa. n = 3 (duplicate samples from three independent cultures/treatments). Unless otherwise indicated, symbols indicate significant difference relative to untreated cells. *, p < 0.05; **, p < 0.01. Scale bar = 100 μm.
NAA and NAAG Promote GSC Growth—Recent metabonomic profiling revealed that NAA and NAAG are significantly reduced in glioma tumors expressing mutant isocitrate dehydrogenase 1 and 2 (IDH1/2) (20). Inasmuch as mutant IDH status is associated with a favorable prognosis in glioma (40), our finding that physiologically relevant doses of NAA and NAAG facilitate GSC growth and differentiation resistance is consistent with the reduction of these metabolites in less aggressive IDH mutant tumors. Despite the prevalence of mutant IDH in oligodendroglioma (41) and proneural GBM (37), the GSCs lines in this study expressed wild-type IDH1 and -2, likely owing to the negative selection of cells with IDH mutations in culture (42). Mutant IDH is hypothesized to drive oncogenesis, in part, by promoting epigenetic dysregulation and the induction of a CpG island methylator phenotype (43); however, why IDH mutations are simultaneously associated with reduced glioma malignancy remains elusive. This study raises the possibility that lower levels of NAA and NAAG could be one contributing factor.

The growth-promoting effects of NAA and NAAG were primarily detected after 5 days of treatment, suggesting a metabolic effect rather than activation of a signal transduction mechanism. In light of the established role for NAA as a source of lipogenic acetate during postnatal myelination (12) and that increased fatty acid synthesis is required to sustain anabolic growth of tumors cells (44), it is likely that NAA hydrolysis exacerbates glioma growth by providing lipogenic acetate. It is unlikely that NAAG signals through metabotropic glutamate receptors as NAAG selectively activates GRM3 (8) and NAAG promoted growth of OG35 GSCs that lacked GRM2, GRM3, GRM5.

FIGURE 6. NAA and NAAG do not promote growth of mesenchymal GBM GSCs and ASPA is differentially expressed in proneural and mesenchymal GBM GSCs. A, PN GBM GSCs up-regulated GFAP expression when cultured in DM for 5 days, whereas mesenchymal (MES) GBM GSC spheres in SCM expressed abundant CD44 that was down-regulated in DM. B, Immunocytochemistry after 6 days in DM demonstrates the cytosolic/perinuclear enrichment of ASPA expression in differentiated PN cells expressing GFAP or nestin. In contrast, MES GBM GSCs in DM exhibit intense nuclear ASPA and were negative for GFAP and nestin but were positive for the cell proliferation marker Ki67. C, MES GBM GSCs proliferate more rapidly than PN GBM GSCs in both SCM and DM. Neither NAA nor NAAG altered growth of MES GBM GSCs in SCM or DM. D, similar to OG GSCs, MES GSCs express the NaDC3 NAA transporter, but not GCP, and GRM2 and GRM5 metabotropic glutamate receptors. Anaplastic oligodendroglioma (Oligo) and GBM tumors served as positive controls and GAPDH as a loading control. E, PN GSCs express ASPA as a 36–38-kDa doublet, whereas MES GSCs primarily express the 36-kDa ASPA and a novel ~24-kDa immunoreactive ASPA species. F, ASPA antibody was incubated with a 5-fold (upper panel) or 10-fold (lower panel) of ASPA protein overnight before Western blot analysis (10 μg of whole cell lysate, 5 day SCM). Immunoreactivity of the 38- and 24-kDa novel ASPA isoforms was completely blocked, whereas reactivity of the putative 36-kDa ASPA was considerably reduced. Blocking of ASPA immunoreactivity (with 10-fold molar ASPA protein overnight) was more efficient for proneural GSCs than mesenchymal GSCs (4 days in DM). G, subcellular fractionation of the PN and MES GSCs grown in DM for 4 days revealed that the 36–38 kDa ASPA primarily partitioned to the cytosolic (C) fraction, whereas the novel immunoreactive ASPA species partitioned exclusively to the nuclear (N) fraction. a.u., arbitrary units. Molecular masses: ASPA = 24 and 36 kDa; CD44 = 95 kDa; GAPDH = 36 kDa; GFAP = 50 kDa; histone H1 = 32 kDa. n = 3 (duplicate samples from three independent cultures/treatments). *, p < 0.05, **p ≤ 0.01. Scale bar = 100 μm.
and GRM5. Although it is possible that NAAG provides acetate via hydrolysis to NAA, OG35 GSC proliferation was increased in the absence of GCPII/III expression. Whether NAAG is converted to NAA by other carboxypeptidases is not known. Our finding that NAA and NAAG produced similar phenotypic effects in Oli-Neu cells and GSCs supports the interpretation that these metabolites function through a common metabolic pathway.

**NAA Metabolism as an Inhibitor of OPC and GSC Differentiation**—This study is consistent with reports from ASPA-deficient mice insofar as increased NAA levels are associated with increased OPC proliferation and diminished oligodendrocyte gene expression (17, 18). However, unlike ASPA-deficient mice, the OPCs and GSCs express ASPA and are capable of NAA catalysis, suggesting that increased NAA catalysis rather than the Accumulation of metabolically inert NAA inhibits OPC differentiation. A major pathological feature of ASPA-deficient mice is the widespread death of immature oligodendrocytes (17). Accordingly, increased OPC proliferation may be a compensatory response to injury arising from defective myelination and diminished oligodendrocyte viability rather than a direct consequence of deficient NAA metabolism.

This study highlights a potential alternative role for NAA in the maintenance of an undifferentiated OPC phenotype. ASPA is expressed by OPCs in mice as early as embryonic day 12.5 (17), and 5–20% of NAA in the postnatal brain is present in proliferating OPCs (45); therefore, OPCs possess the capacity for NAA metabolism independent of myelination. The NAA transporter NaDC3 was expressed in Oli-Neu cells, suggesting that OPCs may take up neuronally derived NAA in vivo. Importantly, we found that NAA inhibited CAMP-mediated Oli-Neu differentiation. Whereas proneural tumors exhibit a gene expression signature that is highly reminiscent of OPCs (37), our observation that NAA and NAAG blunted proneural GBM GSC differentiation supports a role for NAA metabolism in the maintenance of an undifferentiated progenitor-like state.

In the absence of myelogenesis, NAA-defrived acetate/acyetyl-CoA may transition from a lipogenic to an epigenetic function. In OPCs, widespread histone deacetylation is required to silence genes that negatively regulate oligodendrocyte differentiation (46). Thus, increased NAA metabolism may reinforce an acetylated histone state conducive to an OPC phenotype and may, likewise, attenuate proneural GSC differentiation. A relationship between NAA metabolism and histone acetylation is congruent with evidence that increased metabolic flux of other key sources of acetyl-CoA such as citrate (47) and acetylcarboxylamine (48) similarly promote histone acetylation and chromatin remodeling. Moreover, NAA-mediated modulation of acetate bioavailability would add an additional mechanism to regulate histone acetylation underlying OPC differentiation.

**Potential Functions of ASPA in GSC Nuclei**—An unanticipated finding of this study was the detection of novel ASPA isoforms in OG33 and mesenchymal GBM GSC nuclei. Although ASPA is known to undergo cytosolic-nuclear shuttling (13, 14), its nuclear function is not yet established. Coordinated ASPA-mediated NAA catalysis and AceCS1-mediated acetyl-CoA synthesis may contribute to the nuclear pool of acetyl-CoA required to regulate epigenetic chromatin remodeling during oligodendrocyte development. A caveat of this hypothesis, however, is that nuclear ASPA exhibits diminished catalytic activity toward NAA Relative to cytosolic ASPA (13), indicating that, under normal physiological conditions, nuclear ASPA is unlikely to contribute meaningfully to the nuclear acetyl-CoA pool. Alternatively, ASPA may possess a nuclear specific function independent of NAA catalysis. Inasmuch as ASPA-deficient mice show increased histone acetylation, it is possible that ASPA itself functions as a histone deacetylase (18). However, the C-terminal domain of ASPA is so closely juxtaposed with its catalytic site that it limits substrate selectivity to NAA (49). It is, therefore, intriguing that novel lower molecular weight immunoreactive ASPA species were selectively expressed in GSC nuclei. This presents the possibility that post-translational processing or differential mRNA splicing might give rise to ASPA variants with a more accessible catalytic site and confer broader substrate specificity.

Additionally, the mechanisms responsible for ASPA cytosol-nuclear shuttling are not known. It has been suggested that ASPA possesses a non-classical nuclear localization signal near its C terminus (13), but the specific conditions that regulate its nuclear localization are undefined. ASPA did not show overt nuclear regulation in response to NAA and NAAG; however, a lower molecular weight immunoreactive ASPA species was induced by NAA and NAAG in OG33 GSC nuclei, suggesting that its expression is regulated by conditions of heightened acetate load. In contrast, mesenchymal GBM GSCs constitutively expressed a comparable immunoreactive species in the nuclear compartment. Given that GO35 GSCs exhibit a mesenchymal signature similar to OG33 GSCs and the mesenchymal GBM GSCs but did not express a lower molecular weight ASPA isoform, it suggests that an additional regulatory mechanism drives the expression of the novel nuclear ASPA isoform. Thus, the GSC lines used in this study represent a model to better define the nuclear function of ASPA. Furthermore, if nuclear ASPA facilitates GSC malignancy, as is suggested by greater nuclear ASPA immunoreactivity in differentiation-defective mesenchymal GSCs, it would represent a potential target for therapeutic intervention. However, our study demonstrates that the use of NAA or NAAG is not an appropriate therapeutic approach to increase acetate bioavailability; thus, an alternative acetate source is required.

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**REFERENCES**

1. Liu, C., Sage, J. C., Miller, M. R., Verhaak, R. G., Hippenmeyer, S., Vogel, H., Foreman, O., Bronson, R. T., Nishiyama, A., Luo, L., and Zong, H.
signature of malignant glioma reflects accelerated anabolic metabolism. Cancer Res. 72, 5878–5888

39. Phillips, H. S., Kharbanda, S., Chen, R., Forrest, W. F., Soriano, R. H., Wu, T. D., Misra, A., Nigro, J. M., Colman, H., Soroceanu, L., Williams, P. M., Modrusan, Z., Feuerstein, B. G., and Aldape, K. (2006) Molecular sub-classes of high-grade glioma predict prognosis, delineate a pattern of disease progression, and resemble stages in neurogenesis. Cancer Cell 9, 157–173

40. Sanson, M., Marie, Y., Paris, S., Idbaih, A., Laffaire, J., Ducray, F., El Hallani, S., Boisselier, B., Mokhtari, K., Hoang-Xuan, K., and Delattre, J. Y. (2009) Isocitrate dehydrogenase 1 codon 132 mutation is an important prognostic biomarker in gliomas. J. Clin. Oncol. 27, 4150–4154

41. Labussière, M., Idbaih, A., Wang, X. W., Marie, Y., Boisselier, B., Falet, C., Paris, S., Laffaire, J., Carpentier, C., Crinière, E., Ducray, F., El Hallani, S., Mokhtari, K., Hoang-Xuan, K., Delattre, J. Y., and Sanson, M. (2010) All the 1p19q codeleted gliomas are mutated on IDH1 or IDH2. Neurology 74, 1886–1890

42. Piaskowski, S., Bienkowski, M., Stoczynska-Fidelus, E., Stawski, R., Sieruta, M., Szybka, M., Papierz, W., Wolanczyk, M., Jaskolski, D. J., Liberski, P. P., and Rieske, P. (2012) Glioma cells showing IDH1 mutation cannot be propagated in standard cell culture conditions. Br. J. Cancer 104, 968–970

43. Turcan, S., Rohle, D., Goenka, A., Walsh, L. A., Fang, F., Yilmaz, E., Campos, C., Fabius, A. W., Lu, C., Ward, P. S., Thompson, C. B., Kaufman, A., Guryanova, O., Levine, R., Heguy, A., Viale, A., Morris, L. G., Huse, J. T., Mellinghoff, I. K., and Chan, T. A. (2012) IDH1 mutation is sufficient to establish the glioma hypermethylator phenotype. Nature 483, 479–483

44. Menendez, J. A., and Lupu, R. (2007) Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat. Rev. Cancer 7, 763–777

45. Bjartmar, C., Battistuta, J., Terada, N., Dupree, E., and Trapp, B. D. (2002) N-Acetylaspartate is an axon-specific marker of mature white matter in vivo. A biochemical and immunohistochemical study on the rat optic nerve. Ann. Neurol. 51, 51–58

46. Lyssiotis, C. A., Walker, J., Wu, C., Kondo, T., Schultz, P. G., and Wu, X. (2007) Inhibition of histone deacetylase activity induces developmental plasticity in oligodendrocyte precursor cells. Proc. Natl. Acad. Sci. U.S.A. 104, 14982–14987

47. Wellen, K. E., Hatzivassiliou, G., Sachdeva, U. M., Bui, T. V., Cross, J. R., and Thompson, C. B. (2009) ATP-citrate lyase links cellular metabolism to histone acetylation. Science 324, 1076–1080

48. Madiraju, P., Pande, S. V., Prentki, M., and Madiraju, S. R. (2009) Mitochondrial acetyl carnitine provides acetyl groups for nuclear histone acetylation. Epigenetics 4, 399–403

49. Bitto, E., Bingman, C. A., Wesenberg, G. E., McCoy, J. G., and Phillips, G. N., Jr. (2007) Structure of aspartoacylase, the brain enzyme impaired in Canavan disease. Proc. Natl. Acad. Sci. U.S.A. 104, 456–461