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Why Is *N*-Glycolylneuraminic Acid Rare in the Vertebrate Brain?

Leela R.L. Davies and Ajit Varki

Abstract The sialic acids *N*-acetylneuraminic acid (Neu5Ac) and *N*-glycolylneuraminic acid (Neu5Gc) differ by a single oxygen atom and are widely found at the terminal position of glycans on vertebrate cell surfaces. In animals capable of synthesizing Neu5Gc, most tissues and cell types express both sialic acids, in proportions that vary between species. However, it has long been noted that Neu5Gc is consistently expressed at trace to absent levels in the brains of all vertebrates studied to date. Although several reports have claimed to find low levels of Neu5Gc-containing glycans in neural tissue, no study definitively excludes the possibility of contamination with glycans from non-neural cell types. This distribution of a molecule – prominently but variably expressed in extraneural tissues but very low or absent in the brain – is, to our knowledge, unique. The evolutionarily conserved brain-specific suppression of Neu5Gc may indicate that its presence is toxic to this organ; however, no studies to date have directly addressed this very interesting question. Here we provide a historical background to this issue and discuss potential mechanisms causing the suppression of Neu5Gc expression in brain tissue, as well as mechanisms by which Neu5Gc may exert the presumed toxicity. Finally, we discuss future approaches towards understanding the mechanisms and implications of this unusual finding.

Keywords Brain · Central nervous system · *N*-Glycolylneuraminic acid · Sialic acid · Vertebrate

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1 The Evolutionary Origins of Sialic Acids

Sialic acids (Sias) commonly occupy the terminal position on the glycoconjugates that cover all vertebrate cell surfaces, and as such are major determinants of the molecular cell surface phenotype [1]. The term Sia refers to derivatives of *N*-acetylneuraminic acid (Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), and ketodeoxynonulosonic acid (Kdn) (Fig. 1). In 1963, Leonard Warren used the newly developed thiobarbituric acid test to screen for Sias in a wide variety of animals, algae, plants, and fungi [2], detecting them almost throughout the deuterostome lineage of animals, a group that includes vertebrates and so-called "higher" invertebrates. Sias were found to be present in vertebrates, echinoderms, hemichordates, and cephalochordates, although they were not found in urochordates. Outside the deuterostomes, Warren also noted small amounts of Sias within two species of flatworm and the digestive glands of squid and lobster,

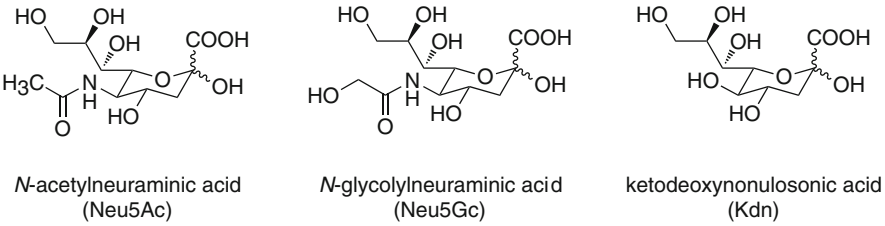

Fig. 1 Structures of the three common sialic acids in vertebrates

but considered these likely to be of dietary origin rather than endogenously synthesized. Based on these and later data, Sias were once considered both unique and universal to the deuterostome lineage [3].

In keeping with this concept, neither Sias nor the genes encoding their biosynthesis can be found in the model protostome *Caenorhabditis elegans* [4]. However, although early studies failed to identify any Sia-containing structures in invertebrates [5], more recent analyses identified small amounts of Neu5Ac in certain protostomes, including in gangliosides of squid and octopus [6], as well as in the slug *Arion lusitanicus* [7]. Furthermore the Drosophila genome encodes a sialyltransferase that functions in one phase of neural development [8]. Thus it seems likely that Sias originated before the deuterostome:protostome split, became prominent only in deuterostomes, and were mostly discarded in protostomes [9].

Outside the animal kingdom there are multiple instances of Neu5Ac production in various species of bacteria [10, 11]. However, the synthetic pathways for Neu5Ac in these species have evolved independently from those of animals, likely as a means of host immune evasion. These bacterial pathways appear to have arisen by convergent evolution, taking advantage of the more ancient pathways for biosynthesis of nonulosonic acids (NulOs), which are likely also the evolutionary precursors of the animal sialic acids [11].

2 Neu5Gc Is a Major Sialic Acid in Many Mammals

Although Kdn can be prominent in some fish, the two major Sias of most mammalian species are Neu5Ac and Neu5Gc. The CMP-Neu5Ac nucleotide sugar donor molecule originates from UDP-GlcNAc, via ManNAc and free Neu5Ac intermediates [3]. CMP-Neu5Ac can then be converted to CMP-Neu5Gc by cytidine monophosphate *N*-acetylneuraminic acid hydroxylase (CMAH). This activity was first identified by Schauer more than 40 years ago [12], then shown to work specifically on CMP-Neu5Ac [13–15], and the enzyme was later cloned and further characterized [16, 17]. Gene knockout studies in mice [18, 19] have indicated that CMAH is likely to be solely responsible for Neu5Gc synthesis. The polypeptide sequence of CMAH is highly conserved across deuterostomes, but appears to be essentially confined to this lineage (Fig. 2).

```
HUMAN          --MGSIEQTTEILLCLSPVEVASLKEGINFFRNKSTGKDYVLYKNKSRLRACKNMCKHQGGLFIKDIEDLAGSC--------------------------  100
CHIMPANZEE     --MGSIEQTTEILLCLSPVEVASLKEGINFFRNKSTGKDYILYKNKSRLRACKNMCKHQGGLFIKDIEDLAGRSVRCTKHNWKLDVSTMKYINPPESFCQ  100
MACAQUE        --MGSTEQTTEILLCLSPVEVANLKEGINFFRNKSTGKDYILYKSKSRLRACKNVCKHQGGLFIKDIEDLAGRSVRCTKHNWKLDVSTMKYINPPESFCQ  100
MOUSE          --MMDRKQTAETLLTLSPAEVANLKEGINFFRNKTTGKEYILYKEKDHLKACKNLCKHQGGLFMKDIEDLDGRSVKCTKHNWKLDVSTMKYINPPGSFCQ  100
RAT            --MMDRKQTAETLLLSLSPADVANLKEGVNFCRNKTTGKEYILYKEKDHLKACKNLCKHQGGLFMRDIEDLDGRSVKCTKHNWKLDVSTMKYTNPPGSFCQ  100
PIG            --MSSIEQTTEILLCLSPAEAANLKEGINFVRNKSTGKDYILFKNKSRLKACKNMCKHQGGLFIKDIEDLNGRSVKCTKHNWKLDVSSMKYINPPGSFCQ  100
COW            --MGSIEQTAELLLCLSPAEVANLKEGINFVRNKSTGKDYILYKSKSLLRACKNMCKHQGGLFIKDIEDLDGRSVRCTKHNWKLDVSTMKYINPPGSFCQ  100
XENOPUS        MEQSNDGQTAHTLLHLASAEVESLKEGITFLRNKESGKNFIIYKNGEELRACKNLCKHQGGTFIKDIEDLGNRTVRCTKHNWKLDVSSMKYVNPPDSFCQ  100
ZEBRAFISH      ----MAAQVSHTVLRLEAEDVRNLKDGINFQKNNKDGKCYIIYKANGELRACRNQCKHQGGLFIKDIEDMDGRTVRCTKHYWKLNVATMQYVNPPDSFMQ  100
SEA URCHIN     ----------------------------------------MYFVSRP-LHVPAAGQTC-----------------VIKCTKHGWKLDAKTMRYTNPPDSFRQ  100

HUMAN          ----------------------------------------------------------------------------------------------------  200
CHIMPANZEE     DELVVEMDENNR-LLLLELNPPNPWDLQPRSPEELAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCQADLIYISHLH  200
MACAQUE        DELVVEMDENNG-LLLLELNPPNPWDSEPRSPEELDFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCQADLIYISHLH  200
MOUSE          DELVIEMDENNG-LSLVELNPPNPWDSDPRSPEELAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLCKADLIYISHMH  200
RAT            DELVVEMDENNG-LCLVELNPPNPWDSDPRSPEELAFGEVQITYLTHACMDLKLGDKRMAFDPWLIGPAFARGWWLLHEPPSDWLERLCKADLIYISHMH  200
PIG            DELVVEKDEENG-VLLLELNPPNPWDSEPRSPEDLAFGEVQITYLTHACMDLKLGDKRMVFDPWLIGPAFARGWWLLHEPPSDWLERLSLADLIYISHMH  200
COW            DELVVEESKENE-LLLLELNPPNPWDSEPRSPEDLAFGEVQITYLTHACMDLKLGDKRMVFDPWLTGPAFARGWWLLHEPPSDWLERLCQADLIYISHMH  200
XENOPUS        DELVIENDDENG-VSLVELSPPNPWDSDPRMAVCLEGGEVQVTYLTHACMDLKLGNKHMVFDPWLIGPAFARGWWLLHEPPCDWLERLCRADLIYISHMH  200
ZEBRAFISH      DELEAVLSETDGSLELLELNPPDPWTAEPREAQDLQAGEITLTYITHACMELKAGERRMMFDPWLTGPAFARGWWLLHEPPKDAMDRLMEADLVYISHMH  200
SEA URCHIN     EQLVSEVDDEGS-MSLIELKPPQPWETDAREKVPLEVGEVKITYLTHACMELNLGGTVMFTDPWLTGPAFARGWWLMHEPPADWLDRLAKADFIYISHVH  200

HUMAN          ----------------------------------------------------------------------------------------------------  300
CHIMPANZEE     SDHLSYPTLKKLAGRRPDIPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP  300
MACAQUE        SDHLSYPTLKKLAGRRPDIPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP  300
MOUSE          SDHLSYPTLKQLSQRRPDIPIYVGDTERPVFWNLDQSGVGLTNINVVPFGIWQQVDKSLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP  300
RAT            SDHLSYPTLKQLSQRRPDIPIYVGDTERPVFWNLNHSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP  300
PIG            SDHLSYPTLKKLAERRPDVPIYVGNTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGVHPEMDTCIIVEYKGHKILHTVDCTRPNGGRLP  300
COW            SDHLSYPTLKKLAGRRPDIPIYVGKTERPVFWNLNQSGVQLTNINVVPFGIWQQVDKNLRFMILMDGIHPEMDTCIIVEYKGHKILNTVDCTRPNGGRLP  300
XENOPUS        SDHLSYPTLKKLSEKRPDIPIYVGKTERPVFWYLDKSGVKLTNINVVPFGIWQEVDENLRFMILMDGVHPEMDTCIIVEYKGNKILNTVDCTRPNGGKLP  300
ZEBRAFISH      SDHLSYPTLQHLSKKRPDIPIYVGNTSRPVFWYLEKSGVNLTNINVVPFGVWQNVDDHLRFMILMDGVHPEMDTCLIVEYKGHMILNTVDCTRPNNGRLP  300
SEA URCHIN     SDHLSYPTLELLSARNPNIPIYVGDTSMPVFCKLKQSGVKLNNINVLKFGKWHEINKDTRFMIMMDGVHPDMDTCILIDYKGHLILNTVDCTNPNGGRLP  300

HUMAN          ----------------------------------------------------------------------------------------------------  400
CHIMPANZEE     MKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKNLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNELNNLIKKNS-DVIT  400
MACAQUE        TKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKAQLVKNLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNELNNLIKKNS-DVIT  400
MOUSE          EKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKAERRKLLNYKAQLVKDLQPRIYCPFAGYFVESHPSDKYIKETNTKNDPNQLNNLIRKNS-DVVT  400
RAT            EKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKAERRKLLNYKAQLVKDLQPRIYCPFAGYFVEAHPSDKYIKETNTKNDPNQLNNLIKKNS-NVVT  400
PIG            MKVALMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKDLQPRIYCPFAGYFVESHPADKYIKETNIKNDPNELNNLIKKNS-EVVT  400
COW            MKVDLMMSDFAGGASGFPMTFSGGKFTEEWKAQFIKTERKKLLNYKARLVKDLQPRIYCPFAGYFVESHPSDKYIKETNIKNDPDELNNLIKKNS-DVLT  400
XENOPUS        TNVALMMSDFAGGASGFPMTFSGGKFTEEWKSQFIKTERKKLLNYKAQLVKDLNPRIYCPFAGYFVEEHPSDKYIKETNLKNDAAELNMLIRNTS-DVVT  400
ZEBRAFISH      HGVDVMMSDFAGGASGFPMTFHGGKYTESWKANFIKNERKKLLNYKAQLVKSLQPKIYCPFAGYFTEAHPSDRYIKETNTKNSPAELNELIRKSCLNTLT  400
SEA URCHIN     IDVDIMLSDFAGGASGFPMNFFGGKYTEEWKGQFIKRERNKLLYYKTQVVRDVNPTVFCPFAGYFVEAHPSDGYIRETNTKNDAASLNALINKYSPEIKT  400

HUMAN          ----------------------------------------------------------------------------------------------------  500
CHIMPANZEE     WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFEPYLEILNAAVGDEIFLHSSWIKEYFTWAGFKDYNLVVRMIETDEDFNPFPGGYDYLVDF  500
MACAQUE        WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFEPYLEILNAAVGDEIFLHSSWIKEYFTWAGFKDYNLVVRMIETDEDFNPFPGGYDYLVDF  500
MOUSE          WTPRPGAVLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFGPYLEILNSAVRDEIFCHSSWIKEYFTWAGFKNYNLVVRMIETDEDFSPFPGGYDYLVDF  500
RAT            WTPRPGATLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFEPYLKTLNSSVRDSIFLHSSWIKEYFTWAGFKNYNLVVRMIETDEDFSPVPGGYDYLVDF  500
PIG            WTPRPGATLDLGRMLKDPTDSKGIVEPPEGTKIYKDSWDFGPYLNILNAAIGDEIFRHSSWIKEYFTWAGFKDYNLVVRMIETDEDFSPLPGGYDYLVDF  500
COW            WTPRPGATLDLGRMLKDPTDSKGIIEPPEGTKIYKDSWDFGPYLKILNAAVGDEIFRHSSWIKEYFTWAGFKDYNLVVRMIETDEDFSPPGGYDYLVDF  500
XENOPUS        WTPKPGAILDLGRLLIDPTDKNGIIDPPPGTKIFKDSWDYDTYLSIHSFSFDDEIFHYPSWIKEYFTWAGFKGYNLVLRMIETDEHFVPLPKGYNYLVDF  500
ZEBRAFISH      WTPLPGSVLDLAVALNNRSNETAITDPPHGTKIYKDNWEFDLYLNQLNASISAEIFKHKHWIQYYYNWAGFRNYNLVIRVIETDDDFQPLNGGFDYLVDF  500
SEA URCHIN     WTPRPGATLDLQKAIQG--DSDYIQESPPGTKVFKDSWDFEKYLNGVNESIEHEIFAYPEWIKVYYKWAKFHSYNLVVRMIETDDDFKDIDGGYNFLIDF  500

HUMAN          ----------------------------------------------------------------------------------------------------  600
CHIMPANZEE     --LDLSFPKERPQREHPYEEIHSRVDVIRHVVKNGLLWDELYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWKSFLMCCEQNGPGILQECKTT----  600
MACAQUE        --LDLSFPKERPQREHPYEEIRSRVDVIRHVVKNGLLWDELYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWRSFLTCCEQNGPGISQECKTT----  600
MOUSE          --LDLSFPKERPSREHPYEEIHSRVDVIRYVVKNGLLWDDLYIGFQTRLLRDPDIYHHLFWNHFQIKLPLTPPNWKSFLMHCD----------------  600
RAT            --LDLSFPKERPSREHPYEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPNWKLFLMRCG----------------  600
PIG            --LDLSFPKERPSREHPYEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPDWKSFLMCSG----------------  600
COW            --LDLSFPKERPSREHPFEEIRSRVDVIRHVVKNGLLWDDLYIGFQTRLQRDPDIYHHLFWNHFQIKLPLTPPDWKSFLMYCG----------------  600
XENOPUS        --LDLSFPTERPERDHPYEEISSRATVIRHVVKHGLLWDDLYIGFQTRIQRNPDIYHHQFWNHFQIKLPLTPPDWKVFLDREKENNATLQNCSIM-----  600
ZEBRAFISH      --LDLSFPTERPEREHAYEEIKNRVNVMRHVVVNGLLWDDLYIGFNNRMSRDPDVYHHKFWNHFQTELPLSAPDWQHFLQICSQTQENGSSNGCSVS---  600
SEA URCHIN     SGLEATFPTERPSREHNYLEIKNRIGVHRQTVLKGLFWDDLYIGFNNQISRTPDTFHYLFWNHMQILLPFEPPNWDEFLDEMKAKNSPKKDVWKPSHAQI  600

HUMAN          ----------------------------------------------------
CHIMPANZEE     ----------------------------------------------------
MACAQUE        ----------------------------------------------------
MOUSE          ----------------------------------------------------
RAT            ----------------------------------------------------
PIG            ----------------------------------------------------
COW            ----------------------------------------------------
XENOPUS        ----------------------------------------------------
ZEBRAFISH      ----------------------------------------------------
SEA URCHIN     TNGKHTVYAGGDNGLKNGGPGAAKPKGGNKGDLMKWLVPIGIAALAAGIMIWRMK
```

Fig. 2 Alignment of vertebrate *CMAH* sequences. Alignment was performed using CLUSTALW [119]

Despite high sequence conservation of vertebrate CMAH, there are some instances of its loss. The human genome is unique among the old world primates in containing a universal deletion spanning exon 6 in the CMAH gene [20, 21]. While one group suggested that this causes an N-terminal truncation of the polypeptide [20], the other showed that an *Alu*-mediated mutation introduces a premature stop codon and highly truncated polypeptide (see Fig. 2), consequently preventing endogenous production of Neu5Gc [21, 22]. However, very low levels of Neu5Gc can be found in human tissues, likely due to metabolic incorporation from the diet [23]. Meanwhile, a recent study has confirmed the previously suspected absence of Neu5Gc in sauropsids – i.e., birds and reptiles – as well as likely in monotremes [24]. In keeping with this, no sequence strongly homologous to vertebrate *CMAH* can be found in any of the sauropsid genomes so far sequenced. Although some Neu5Gc was noted in one lizard and one bird – the green basilisk and the budgerigar – this was also suspected to originate from food [24]. Some strains of dogs and cats are deficient in erythrocyte Neu5Gc [25, 26]; it is as yet unclear whether this is caused by genomic *CMAH* mutations. Changes in the *CMAH* promoter, however, have been identified in some cats that appear to define the feline type B blood group that expresses solely Neu5Ac on erythrocytes [27].

There are many consequences to the loss of Neu5Gc production in a mammal. A mouse model of the human *CMAH* mutation, for example, exhibits numerous phenotypes, including delayed wound healing and age-dependent hearing loss [19], heightened B cell responses [18, 28], and a tendency for decreased insulin production [29]. Like this mouse model, vertebrate lineages that have lost functional *CMAH* are viable and fertile. However, Neu5Gc loss can cause relative infertility with wild-type animals, because of female anti-Neu5Gc antibodies that attack Neu5Gc-positive sperm or embryos [30].

3 Rarity of Neu5Gc in the Vertebrate Brain

Despite a universally high concentration of Neu5Ac in the brain, Neu5Gc has long been noted to be rare in the central nervous system (CNS) [3, 31]. Although it is well known in the field that Neu5Gc is rare in the vertebrate brain, we were unable to ascertain when and where this observation was first recorded in writing.

Unlike the highly variable levels of Neu5Gc found in all other tissues, the suppression of brain Neu5Gc expression is remarkably conserved across all vertebrates that have been studied to date (see Table 1, reproduced from [32]). The highest published fraction of Neu5Gc in brain tissue is 10% in a sample of adult bovine neocortex and 5% in calf [33]. However, other published studies of bovine brain report much lower fractions of under 2% [31, 34, 35]. Notably, even species that otherwise have a substantial fraction of their total Sias as Neu5Gc in many non-neural tissues maintain this suppression of Neu5Gc expression within the brain.

Table 1 Distribution of Neu5Gc in vertebrate tissues

Species	Serum	RBC	Submaxillary gland	Liver	Kidney	Milk	Brain
Human	–	–	nr	nr	–	–	–
Chimpanzee	nr	++	nr	+	+	+[a]	trace[b]
Macaque	+	+	nr	nr	nr	+	–
Mouse	+	+[b]	–	++	nr	nr	trace[b]
Rat	+	+[b]	+	+	+	nr	trace[b]
Rabbit	trace	+	nr	–	+	nr	–
Pig	nr	++	++	+	+	nr	trace[b]
Cow	++	++	+	nr	++	trace	trace[b]
Sheep	+	++	trace	+	+	++	trace
Elephant Afr	nr	nr	nr	++[a]	nr	+	nr
Elephant Asian	nr	nr	nr	++[a]	nr	–	nr
Dolphin	nr	nr	nr	++[a]	++	+[a]	trace[a]
Horse	+	++	trace	–	+	nr	trace
Chicken	–	–	–	–	–	–	–
Xenopus	nr	nr	nr	nr	nr	nr	–

The Neu5Gc fraction of total Sias in tissues was compared across vertebrates. This table combines data from the literature with that obtained from samples studied in our laboratory. Neu5Ac and Neu5Gc fractions of samples in our lab were determined by total acid hydrolysis of tissue lysate followed by DMB-HPLC. Conserved suppression of Neu5Gc in the brain is unusual among vertebrate tissues

++: major fraction; +: minor fraction; –: absent; trace: present at 0.8–3%; nr: not reported

[a]Data from our laboratory

[b]Published data confirmed in our laboratory

To our knowledge, no other molecule exhibits such an unusual tissue distribution. The evolutionary conservation of strong CNS suppression seems to indicate that maintaining very low levels of Neu5Gc in the CNS is very important.

4 Widely Variable Expression of Neu5Gc in Non-neural Tissues of CMAH-Positive Mammals

The conserved regulation of Neu5Gc expression in a given tissue across species is found only in the brain. As shown in Table 1, the Neu5Gc fraction of total sialic acid otherwise varies widely both across tissues and across species. To take the horse as an example, the fraction of total sialic acid that is Neu5Gc is as low as 1–2% in submaxillary mucin [31] but almost 100% on erythrocytes [36, 37]. This remarkable variability can also be found in interspecies comparisons within a single tissue. For example, in pigs, 90% of sialic acid in submaxillary mucin is Neu5Gc [38], while in cows, it is only 15% [31]; in sheep and horses it is lower still at only 1–3% [31]. Meanwhile, the fraction of Neu5Gc on erythrocytes from these species

shows no correlation to that found on the mucins. Thus Neu5Gc expression appears to be extremely dynamic in its regulation and evolving rapidly in all tissues other than the brain.

Our understanding of the regulatory mechanisms establishing the fraction of Neu5Gc in a given cell type is incomplete. One study of the developmental regulation of Neu5Gc in rat tissues found that CMAH enzymatic activity is an imperfect correlate to the level of Neu5Gc, suggesting that other mechanisms may influence the presence of Neu5Gc in sialylated glycans [39]. A more recent study of porcine tissues found that CMAH enzyme activity correlated reasonably well with Neu5Gc levels; however, in lung and heart tissues a much larger amount of immunoreactive protein was present, suggesting that modification of CMAH may influence its activity [40]. Nevertheless, although other factors may be at play, CMAH expression appears to be the major determinant of Neu5Gc levels.

The factors that determine the optimal ratio of Neu5Gc to Neu5Ac for a given tissue in a given animal are not entirely clear; however, it is easy to imagine the types of evolutionary selection pressures that may have had an influence. Sialic acid-binding lectins, whether pathogenic or endogenous, typically prefer either Neu5Ac or Neu5Gc, and these may affect the balance of Neu5Ac and Neu5Gc presence. Indeed, the Neu5Ac binding preference of ancestral *Plasmodium* species is postulated to be the impetus for fixation of the *CMAH* deletion in humans [41–43]. Siglec-2 (CD22), an endogenous immune modulator found on B cells, preferentially binds Neu5Gc in mice [44], but will bind either Neu5Ac or Neu5Gc in humans and great apes [45]. Recent work on a mouse model of muscular dystrophy shows that the absence of Neu5Gc is required to produce a severe, human-like phenotype, indicating that the presence of Neu5Gc may be important in normal muscle physiology [46]. Thus both pathogenic and endogenous selective pressures may influence the balance of Neu5Gc and Neu5Ac that is ultimately expressed within a given tissue. The data make it clear, however, that most tissues do not have an intrinsic requirement for a specific proportion of Neu5Gc in total sialic acids, let alone an exclusive preference for Neu5Ac or Neu5Gc.

5 Sialic Acids in the Vertebrate Brain

In seeking to understand why the vertebrate CNS suppresses the presence of Neu5Gc so consistently, it is important to consider the existing knowledge of CNS sialic acid biology. The brain contains more Sias than any other tissue [3, 31, 47]. In fact it was from brain tissue that Ernst Klenk extracted what he then called “neuraminic acid” in 1939, only a few years after the first isolation of sialic acids from salivary gland [31]. Interestingly, Neu5Ac content has been found to be approximately 30% higher in the left hemisphere than in the right hemisphere of a chimpanzee, suggesting that brain Sia concentration may correlate with neurological function or hemispheric dominance [48]. It has also been postulated that sialic acid from dietary sources can increase the Sia concentration in the developing

brain, playing an important role in learning and memory [49, 50]. Conversely, however, other studies of the incorporation of maternal dietary Neu5Gc into pups in utero have not found uptake of Neu5Gc into brain tissue [51], suggesting that dietary Neu5Ac and Neu5Gc may differ in their ability to be incorporated into brain glycans. It is clear, however, that Sias are extremely important within the brain.

As is the case elsewhere in the body, Neu5Ac in the brain can be found on N- and O-linked glycoproteins and on glycolipids; however, the distribution is somewhat different. It has been estimated that about 65% of brain sialic acid is on gangliosides, 32% on glycoproteins, and only 3% remains unbound [52]. This preponderance of ganglioside-bound, rather than glycoprotein-bound, sialic acid is unique among all tissues [49]. The brain also contains several characteristic sialoglycoconjugates, which we will briefly consider here.

5.1 Polysialic Acid

Polysialic acid (polySia) is an unusual posttranslational modification found on a few mammalian proteins as well as in the jelly coat of certain fish eggs, the voltage-gated sodium channel of eel, and the capsules of certain pathogenic bacteria. The most well studied carrier of polySia in the brain is the neural cell adhesion molecule (NCAM). Brain polySia is composed of long α2-8-linked polymers of Neu5Ac synthesized by two polysialyltransferases, ST8SiaII and ST8SiaIV, within the Golgi apparatus [53–55].

There is significant developmental regulation of the expression of polysialylated NCAM (also called embryonic NCAM or PSA-NCAM); in mice, very high levels occur in early postnatal life, mediated primarily by ST8SiaII, that drop to low adult levels by about 3 weeks of age [56]. Polysialylated NCAM plays a wide range of crucial roles throughout development affecting migration of neural progenitors, neurite outgrowth, and formation of appropriate synapses [57]. These roles are mediated through two mechanisms. Through the formation of a highly hydrated anionic cloud, polySia attenuates the adhesive properties of NCAM [58] and other adhesion molecules. PolySia also mediates other effects, specifically sensitizing cells to brain derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF-2) through direct interaction [59–62]. Later in development, downregulation of polySia has been found to be necessary for myelination of axons [63, 64]. In adult mammals, polySia is expressed at much lower levels throughout the brain, but can still be found in areas such as the hippocampus that are associated with adult plasticity [65].

Although polySia-NCAM is the most well characterized polysialylated glycoprotein, polySia has been identified on other vertebrate carrier proteins. Within the brain, it has been found on the synaptic cell adhesion molecule (SynCAM) on a specialized subset of glial cells, where it is thought to modulate synaptic formation [66], and has been identified in rat brain on the voltage-gated sodium

channel [67]. Although one study claimed to find polySia on neural podocalyxin, this was actually an erroneous use of the term to describe a highly sialylated protein [68].

5.2 Oligosialic Acid

A related but less well understood structure is oligosialic acid, i.e., two to four residues of α2-8-linked Neu5Ac. Oligosialic acids are enriched in the brains of both embryonic and adult pigs [69]. A specific trisialic acid epitope found on mouse brain glycoproteins appears, like polySia, to be developmentally regulated [70]. However, the functions of oligosialic acids remain relatively unknown. Notably, the oligosialic acid epitope is recognized by Siglec-11, a protein altered by gene conversion in humans, that is found on tissue macrophages and shows human-specific expression exclusively in microglia in the brain [71–73]. Although its function is not well understood, Siglec-11 was recently found to decrease the transcription of inflammatory mediators and thus reduce neurotoxicity induced by bacterial lipopolysaccharide (LPS) [74].

5.3 Gangliosides

Gangliosides are sialic acid-containing glycosphingolipids expressed throughout the body but most highly in brain tissue. In mammals, the simple gangliosides GD3 and GM3 predominate early in development throughout formation of the neural tube and neural stem cell proliferation, but this profile changes by the major period of neurogenesis [75]. In adult mammals, GM1, GD1a, GD1b, and GT1b together comprise over 85% of total brain gangliosides in pigs and 95% in humans [76]. These were found to be most prevalent in the neuropil surrounding neurons, suggesting an enrichment in neurons, particularly in synaptic membranes, rather than in glial cells [77].

Brain gangliosides have been proposed to be important in neurogenesis, neurite outgrowth, synaptogenesis, and synaptic function; however, the mechanisms underlying these roles have not been completely elucidated. Abolishing all ganglioside production by knockout of glucosylceramide synthase causes abrupt embryonic lethality after division of the primitive germ layers [78]. Neural-specific disruption of glucosylceramide synthase in mice produces live births, but the animals have severe neural abnormalities and die at 2–3 weeks of age, suggesting a critical impairment of appropriate brain maturation [79]. However, mice lacking only complex gangliosides were reported not to have any major abnormalities in histology or behavior, only a decrease in neural conduction velocity at 10 weeks of age [80]. Thus it has been hypothesized that gangliosides play a role in neural membrane function, such as signaling, conduction, or stability [81]. However, another group

found that mice lacking complex gangliosides were found by 16 weeks of age to exhibit more severe phenotypes, including progressive axonal degeneration of optic and sciatic nerves, as well as decreased central myelination [82].

The phenotype found in this study was noted to be similar to that caused by a deficiency of another important sialic acid-binding lectin, myelin-associated glycoprotein (MAG, Siglec-4), suggesting a common pathway for these molecules [82]. MAG is found periaxonally on Schwann cells and oligodendrocytes in both peripheral and central nervous systems. Notably, it preferentially binds the epitope Neu5Acα2-3Galβ1-3GalNAc [83], found on the complex neuronal gangliosides GD1a and GT1. Deficiency of MAG is associated with progressive signs of peripheral demyelination and axonal degeneration, similar to demyelinating peripheral neuropathies found in human patients [84, 85]. Additionally, the binding of MAG to gangliosides has been implicated in inhibition of neuronal regeneration after injury [86].

6 Claims for Presence of Neu5Gc in Neural Tissues

A number of studies have claimed to find small amounts of Neu5Gc-containing glycans in neural cells and tissues. It is worth considering these studies in some detail.

6.1 Tumor Gangliosides

Increased Neu5Gc expression is a feature of human cancers [87], and it might be expected that neural tumors would express Neu5Gc. An extensive characterization of mouse brain tumor gangliosides by Seyfried and colleagues has indeed repeatedly found Neu5Gc present in these gangliosides. However, Neu5Gc is only found when the tumor cells are injected subcutaneously into the flanks of mice, and not when the tumor cells are cultured independently, suggesting that the observed Neu5Gc is exogenous [88]. Subcutaneous injection of an experimental ependymoma incapable of synthesizing endogenous GM2(Neu5Gc) results in a tumor that contains GM2 (Neu5Gc) as well other gangliosides suggestive of the presence of Neu5Gc-expressing macrophages [89]. These Neu5Gc-containing gangliosides are still found when the cells are injected into mice with severe combined immune deficiency (SCID), which have no B or T lymphocytes, further suggesting that the gangliosides present do originate from tissue macrophages and/or from metabolic uptake by the tumor cells [90]. To date, no characterization of naturally occurring neural tumors has demonstrated endogenous Neu5Gc synthesis.

6.2 Normal CNS Gangliosides

There have, however, been a number of claims that Neu5Gc exists in normal brain gangliosides of some mammals. In 1970, Yu and Ledeen used gas–liquid chromatography to analyze sialic acids in the brain gangliosides of several species. Although the specific gangliosides were not defined, this study found Neu5Gc at 1–2% of total ganglioside sialic acids in ox, bull, and calf, 0.1–0.2% in pig and sheep, 0.4% in goldfish, but undetectable in rat, rabbit, frog, and chicken [35]. More recently, a study of cetacean brain gangliosides found low percentages (<2%) of Neu5Gc in the total ganglioside content of cerebrum and cerebellum of three toothed whales (killer whale, Dall's porpoise, and sperm whale). No Neu5Gc was found to be present, however, in the brains of other members of the dolphin family or baleen whale species (minke whale and Bryde's whale) [91].

Other studies have been able to identify specific gangliosides containing Neu5Gc in the brains of certain species. The first and most common such ganglioside to be identified was GD1a containing both Neu5Ac and Neu5Gc, initially estimated to account for 1% of total ganglioside-bound sialic acids in bovine brain [92]. However, a later estimate put the fraction of GD1a(Neu5Ac/Neu5Gc) much lower, at only 0.1% of total bovine brain ganglioside [93]. Using a two-step DEAE-Sepharose and TLC approach to increase resolution, Iwamori and Nagai also identified Neu5Gc-containing GD1a, as well as GM1, in the brain of cow; these were not found in brains of human, chicken, cat, rabbit, rat, or dog [94]. Another early study of calf and pig brain tentatively identified two unknown gangliosides as Neu5Gc-containing GD1a as well as GM3 [95]. GM3(Neu5Gc) has also been identified in equine brain, where Neu5Gc was found to comprise 18% of total GM3 Sias [96]. Finally, Neu5Gc-containing GT1b has been identified in extracts from bovine brain [97]. There are, therefore, quite a variety of gangliosides identified in these reports.

A major challenge in interpreting all such studies is that they did not separate neural cells, i.e., neurons and glia, from the endothelial cells and blood contents that run throughout the brain. Are the Neu5Gc-containing gangliosides that are being isolated in these experiments truly neural, or do they instead arise from the non-neural vasculature? The only study to address this question directly is an examination of horse brain [96]. The authors allow that the presence of endothelial cells may affect their results; however, they did determine that the lipid composition associated with this ganglioside was 60% 18:0, a feature characteristic of brain gangliosides as compared to aortic endothelial cells, which express a wide range of fatty acids [96].

A comparison of the types of gangliosides found in neural and endothelial tissues may also help to clarify the results of these studies. It can be expected that brain microvasculature, comprising the blood–brain barrier, may have different characteristics than endothelium from other tissues; however, unfortunately, few characterizations of the gangliosides of these cells have been done. Immortalized and cultured human cerebromicrovascular endothelial cells have been shown to

express GM3 (62%), GM2 (18%), GM1 (3%), and GD1a (15%) as the major gangliosides [98]. A similar cell line has GM3 and LM1, with small amounts of GM1, GD1a, GD1b, and GT1b [99]. Cultured microvascular endothelial cells from bovine brain also express GM3 as the major ganglioside component, with approximately 58% of GM3 containing Neu5Gc [100], although it is important to note that Neu5Gc in these cultured cells could have originated from components of the growth medium. As noted above, the major brain gangliosides are GM1, GD1a, GD1b, and GT1b. Thus, the above identification in horse brain of GM3(Neu5Gc) as a neural ganglioside is puzzling, as GM3 is a very minor component of brain gangliosides but a major fraction of cerebromicrovascular cells. Unfortunately, the significant overlap in expression of the remaining gangliosides in the two tissues makes it impossible to clarify the published identification of Neu5Gc in GM1, GD1a, and GT1b any further.

The fact that all published studies purporting to find Neu5Gc in brain gangliosides involve a group of closely related species – the even- and odd-toed ungulates and the cetaceans – may support the validity of their findings. Perhaps there is an evolutionary adaptation to allow low percentages of Neu5Gc within the brains of these animals. However, these species also happen to be mammals with a large enough amount of brain tissue to detect very minor ganglioside fractions, and the observation may thus result from a sampling bias.

Overall, the data on Neu5Gc in CNS gangliosides are quite challenging to interpret. While it is impossible to rule out completely endothelial and/or blood contamination in any case, it is also impossible to rule out a low level of Neu5Gc presence in neural cells. Further work will be necessary to clarify this issue fully.

6.3 Normal CNS Glycoproteins

Although much work has been done to characterize nervous system gangliosides, to our knowledge no study has so far identified Neu5Gc on a brain glycoprotein. It is interesting to note here that polysialyltransferases are able to incorporate a number of unnatural sialic acids into polySia [101, 102]. Recently, our laboratory has demonstrated that cells from a murine neuroblastoma line are similarly able to incorporate Neu5Gc into endogenous polySia [32]. Although polymers of Neu5Gc are found in the glycoproteins of the eggs of salmonid fish [103], Neu5Gc has never been reported in mammalian polysialic acid, neural or otherwise.

6.4 PNS Glycoconjugates

It is possible that the central nervous system suppression of CMAH and Neu5Gc does not extend to the peripheral nervous system (PNS). Very little work has examined this question. A study of bovine spinal motor neuron gangliosides that

reacted to serum antibodies from patients with Guillain–Barré syndrome identified two unknown gangliosides that the authors suggested, although did not show definitively, were GD1a containing one or two Neu5Gc residues [104]. Additionally, a membrane mixture of the noradrenergic vesicles from bovine sympathetic nerve endings was found to contain close to 50% Neu5Gc [105]. It is therefore quite possible that Neu5Gc is expressed without consequence in peripheral nerves.

It remains to be seen whether Neu5Gc retains a small presence in – or is completely absent from – the vertebrate CNS. The repeated finding of Neu5Gc in characterizations of neural gangliosides may indicate a true neural presence. Regardless, the very difficulty of detection and interpretation of these studies makes it clear that any Neu5Gc present is maintained at an extremely low level within the vertebrate brain. Further studies are needed, including in situ staining and/or cell-sorted analyses.

7 Possible Mechanisms for the Rarity of Neu5Gc in the Brain

The published record makes it clear that, for as yet unknown reasons, vertebrate brains have very low levels of Neu5Gc, regardless of the levels in other cell types in the same organism. There are a number of possible mechanisms responsible for this suppression, which has apparently persisted for hundreds of millions of years of vertebrate evolution. The most likely explanation is simply transcriptional repression of *CMAH*. Indeed, northern blot analysis of *Cmah* in mouse tissues detected no message in brain [16]. Further, the Allen Brain Atlas, which stores images of in situ hybridization to mouse brain slices, shows no *Cmah* signal throughout the brain (Allen Mouse Brain Atlas [Internet]. Seattle (WA): Allen Institute for Brain Science. ©2009. Available from: http://mouse.brain-map.org). Microarray analysis of *Cmah* cDNA in mouse brain gives a low but detectable level [106], although again endothelial contamination cannot be ruled out in whole brain extracts. Absence of *CMAH* transcript from brain has also been found by RT-PCR analysis of pig tissues [107]. Interestingly, human tissue mRNA microarrays give similar results [108], indicating that *CMAH* continues to be transcriptionally regulated long after its pseudogenization in the human lineage.

However, the pathway by which *CMAH* might be transcriptionally repressed is completely unknown. It may be induced by an extracellular factor, either soluble or membrane-bound. Conversely, it could be cell-intrinsic, a feature of neuronal/glial differentiation. The latter explanation is supported by the studies of gangliosides of murine neural tumor cells, which remain unable to synthesize Neu5Gc even when grown in culture away from their normal extracellular environment [88, 89]. Further, this same group found Neu5Gc-containing gangliosides in murine solid tumors of neural origin grown intracerebrally and in brain metastases of subcutaneously grown tumors, demonstrating that although neural cells do not express

Neu5Gc, inflammatory cells can maintain Neu5Gc expression even within the cerebral compartment [109]. It is therefore probable that *CMAH* downregulation is a characteristic of the neural lineage.

Of course, other mechanisms may influence Neu5Gc levels in neural cell types. CMAH may be further regulated at the level of mRNA stability, translational rate, and/or by posttranslational modification. Additionally, it cannot be ruled out that there may be enhanced mechanisms for eliminating Neu5Gc after it is synthesized, either within the neural cell or on the cell surface. The apparent evidence that dietary Neu5Ac, but not Neu5Gc, is incorporated into the developing brain may indicate that such an elimination is in fact taking place [50, 51]. Regardless, we can think of no other molecule that has such an unusual distribution amongst vertebrates: expressed at widely variable levels throughout extra-neural tissues, and yet always at very low levels in the brain. Whatever the mechanism, the neural regulation of Neu5Gc is apparently very tightly controlled.

8 Is Neu5Gc "Toxic" to the Vertebrate Brain?

The unusual picture of Neu5Gc distribution is suspicious for harmful effects of Neu5Gc on the brain. The degree of suppression is remarkable, with the Neu5Gc fraction being no more than about 2% (see Table 1). This finding implies that Neu5Gc is quite severely detrimental to the brain. To date, no studies have examined whether this presumed toxicity of Neu5Gc does in fact occur.

It is interesting to consider whether toxicity might be cell-intrinsic or extrinsic – that is, whether the toxicity targets a unique property of individual neural cells or only exerts its effects on the overall organ of the CNS. There is significant evidence to suggest that the latter is the case. Standard cell culture methods for all cell lines, including neurons, frequently involve fetal calf serum, a source of Neu5Gc. It has been shown that cells grown in such conditions will take up free Neu5Gc by macropinocytosis and incorporate it into glycans [110]. This has been shown to be true of human embryonic stem cells as well [111]. Neurons are routinely grown and differentiated in culture within such Neu5Gc-rich conditions. It is thus likely that Neu5Gc is toxic only on an intercellular level within the whole brain.

Despite these indications of a detrimental effect of Neu5Gc to the brain, it would be an unexpected finding. Neu5Gc is expressed to no ill effect throughout a wide range of other tissues, so such an effect would have to target neural tissue specifically. Even more puzzlingly, Neu5Ac, which differs only by a single oxygen atom, is a highly prevalent and critically important molecule in brain glycoconjugates. It will be fascinating to explore this question in future work. A number of mechanisms by which Neu5Gc might exert toxicity in the CNS are considered in the next section.

9 Possible Mechanisms for the Presumed Toxicity of Neu5Gc in the Brain

9.1 Biophysical Properties

Neu5Ac and Neu5Gc have some differences in chemical properties. Notably, the presence of the additional hydroxyl group on Neu5Gc may alter its pK_a from that of Neu5Ac [112]. This structural change also increases the hydrophilicity of Neu5Gc. These effects are minor when considered on the level of individual molecules. However, a mammalian erythrocyte, for example, contains many millions of surface sialic acid residues [113] and neural cells likely have even more. The overall effect of replacing Neu5Ac with Neu5Gc may thus have major effects on the surface charge and/or hydropathicity of a neural cell as a whole.

9.2 Differentiation

It is possible that the toxic effect is one to which neural progenitors or differentiating cells are primarily vulnerable. However, there is no evidence that CMAH and Neu5Gc are expressed in these immature progenitors, nor that their presence is toxic to neural differentiation. In fact, cultured neurons can be differentiated in the laboratory with Neu5Gc-rich fetal calf serum with no apparent detriment. Further, this explanation would also not account for the continued CNS suppression of *CMAH* into adulthood. It therefore seems somewhat unlikely that the presumed toxicity of Neu5Gc is mediated at the level of differentiation.

9.3 Sialoglycoconjugates

The presence of Neu5Gc in one of the sialic acid-containing molecules previously discussed may cause a detrimental effect. Although most sialyltransferases exhibit a preference for CMP-Neu5Ac or CMP-Neu5Gc, most will utilize either substrate if available [114]. Does Neu5Gc incorporation into a characteristic brain sialoglycoconjugate cause toxic aberrant function? Possibilities include polysialic acid; one study found polymers of Neu5Gc to be less effective at binding BDNF, although the authors acknowledged these were also shorter than the control Neu5Ac polymers [60]. The conversion of Neu5Ac to Neu5Gc in neuroblastoma–glioma hybrid cells also abrogates MAG binding [115].

Alternatively, perhaps Neu5Gc inhibits synthetic or degradative enzymes of brain sialoglycoconjugates, mediating toxicity not by affecting the function but by blocking the normal turnover of Neu5Ac-containing glycans. Indeed, a recent

paper from our own laboratory demonstrated that Neu5Gc is relatively resistant to degradation by sialidase when present in the α2-8 linkage common in brain glycans, including polySia [32]. This phenomenon may allow for a very small fraction of Neu5Gc to be highly detrimental to the brain, as an entire chain of polySia can be rendered resistant to breakdown by the presence of a terminal Neu5Gc. To date, this is the only hypothesis explaining a potential mechanism for Neu5Gc toxicity.

9.4 Alternative Role of CMAH

Lastly, although speculative, it is conceivable that CMAH has an additional role aside from converting Neu5Ac into Neu5Gc, and that it is this alternative function that requires suppression to avoid toxicity. In fact, a recent study of human stem cells suggested that human CMAH, although inactive as a hydroxylase, increases cellular uptake of exogenous Neu5Gc and decreases Wnt/β-catenin signaling [116]. However, this work depended heavily on an incorrectly reported N-truncated cDNA sequence [20]; the full-length human cDNA actually contains a stop codon upstream of the incorrect start site used [21]. Moreover, the raw data presented do not seem to support the claimed correlations. With the exception of this study, there is no work examining the possibility of an alternative role for CMAH. However, the continued tissue-dependent regulation of *CMAH* mRNA in humans may indicate that one exists. Perhaps this alternative role is the true mediator of neural toxicity.

The fact that makes few of these explanations entirely satisfactory is that Neu5Gc is not merely low in the brain, but that it is almost – perhaps completely – nonexistent. Any model of Neu5Gc toxicity in the vertebrate CNS will have to explain an evolutionarily selective effect requiring that Neu5Gc levels remain below 1–2% of total brain Sias. We believe this feature makes an inhibition of degradation hypothesis the only viable possibility at present. The fact that α2-8 linked Sias are uncommon outside the nervous system and widely distributed in brain glycoconjugates supports this notion [69].

10 Evolutionary Implications

If Neu5Gc expression is truly toxic to the vertebrate CNS, the expression or lack thereof of CMAH in a given animal may have implications for its neural evolution. The ancestral origins of CMAH are unfortunately not well understood, yet they may be important to our understanding of Neu5Gc in modern vertebrates. *N*-Acetylmuramic acid hydroxylase (namH) has been identified in certain species of *Mycobacteria*, where it converts UDP-*N*-acetylmuramic acid to UDP-*N*-glycolylmuramic acid [117]. Although the homology between namH and murine CMAH is only 12% at the peptide level, namH remains the most likely explanation as to the ancestral source of vertebrate CMAH. Unlike vertebrates, certain early

sialic acid-expressing invertebrates, such as the echinoderms, actually express a predominance of Neu5Gc [2], perhaps indicating that the development of CMAH and Neu5Gc was of importance in early lineages.

Within vertebrates, however, species that have since lost Neu5Gc may have gained some neural advantage in the bargain. Admittedly, it is difficult to see what benefit the brains of birds and reptiles have gained from losing Neu5Gc. However, the concentration of sialic acid in brain tissue in humans is reported to be extremely high – two- to fourfold that of most other mammalian species, and slightly increased over that of chimpanzees [48, 118]. Particularly considering the critical role that polysialic acid plays in neural plasticity, outgrowth, and myelination, it is tempting to speculate that the outright loss of neural Neu5Gc in an already sialic acid-rich brain may have eliminated a residual structural constraint and enabled the evolution of a larger, more complex, and more plastic brain in humans.

11 Conclusions and Future Prospects

The rarity of Neu5Gc in the vertebrate brain is certainly a fascinating observation. The literature to date indicates that this absence is highly conserved, with no animal expressing more than 1–2% Neu5Gc on neural gangliosides. Given the prominent sialylation of important neural glycoconjugates, such as gangliosides and NCAM, the striking absence of CMAH and Neu5Gc from neural cells is highly unusual. Although these findings are suspicious for a detrimental effect of Neu5Gc on the CNS, there is a dearth of studies examining this question. A number of questions will therefore need to be addressed in future work.

11.1 How Conserved Is Neu5Gc Suppression?

It is not yet clear whether some species are able to maintain a low percentage of Neu5Gc expression within neural tissue. It will be interesting to see whether some Neu5Gc does persist in the neural tissue of certain species, and to consider what implications this may have for those species. Additionally, no studies to date have observed whether there is similar suppression of Neu5Gc expression in the nervous systems of invertebrate deuterostomes, such as starfish and sea urchin.

11.2 What Is the Precise Localization of Residual Neu5Gc Within the CNS?

From existing studies it cannot be established whether trace Neu5Gc presence is confined to endothelial and blood cells or extends across the blood brain barrier. If it

exists within the CNS itself, cell-specific analyses will be necessary to determine whether all or only some cell types are able to express it. We are currently studying this issue.

11.3 What Is the Developmental Regulation of CMAH and Neu5Gc?

No study to date has examined whether Neu5Gc is endogenously expressed in neural progenitor cells or embryonic stem cells. This knowledge may help to clarify the mechanisms and the significance of Neu5Gc absence in mature brain cells.

11.4 What Mechanisms Cause the Suppression of CMAH Expression and Neu5Gc Production in the CNS?

This question will be particularly interesting given the unique distribution of CMAH, which exhibits dynamic regulation in non-neural tissues but strict neural suppression. Rigorous examination of the transcriptional regulation of *CMAH* is a necessary initial step.

11.5 Does the Presence of CMAH and/or Neu5Gc Truly Have Detrimental Effects on the Vertebrate Brain?

Animal models are needed to address this question since, as previously discussed, Neu5Gc is not toxic in cell culture. It will further be necessary to study whole brain tissue as well as individual cells. It is worth examining overexpression of CMAH and Neu5Gc separately in this work, as an additional role of CMAH mRNA or protein has not been ruled out.

These questions are critical in determining whether the repeatedly observed "smoke" of CNS rarity of Neu5Gc truly represents clues to a real "fire." In our ongoing and future work we are beginning to explore this decades-old unexplained observation, using a variety of methods. Our preliminary studies are promising in this regard. The evidence to date is certainly intriguing, and further investigation may help our understanding of Sias and the CNS.

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