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*Dm*SAS Is Required for Sialic Acid Biosynthesis in Cultured *Drosophila* Third Instar Larvae CNS neurons

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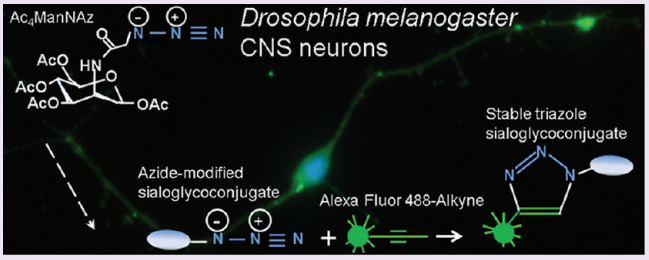
Supporting Information

ABSTRACT: Sialylation is an important carbohydrate modification of glycoconjugates that has been shown to modulate many cellular/molecular interactions in vertebrates. In *Drosophila melanogaster* (*Dm*), using sequence homology, several enzymes of the sialylation pathway have been cloned and their function tested in expression systems. Here we investigated whether sialic acid incorporation in cultured *Dm* central nervous system (CNS) neurons required endogenously expressed *Dm* sialic acid synthase (*Dm*SAS). We compared neurons derived from wild type *Dm* larvae with those containing a *Dm*SAS mutation (148 bp deletion). The ability of these cells to produce Sia5NAz (sialic acid form) from Ac_4ManNAz (azide-derivatized *N*-acetylmannosamine) and incorporate it into their glycoconjugates was measured by tagging the azide group of Sia5NAz with fluorescent agents *via* Click-iT chemistry. We found that most of the wild type *Dm* CNS neurons incorporated Sia5NAz into their glycoconjugates. Sialic acid incorporation was higher at the soma than at the neurite and could also be detected at perinuclear regions and the plasma membrane. In contrast, neurons from the *Dm*SAS mutant did not incorporate Sia5NAz unless *Dm*SAS was reintroduced (rescue mutant). Most of the neurons expressed α2,6-sialyltransferase. These results confirm that the mutation was a null mutation and that no redundant sialic acid biosynthetic activity exists in *Dm* cells, *i.e.*, there is only one *Dm*SAS. They also provide the strongest proof to date that *Dm*SAS is a key enzyme in the biosynthesis of sialic acids in *Dm* CNS neurons, and the observed subcellular distribution of the newly synthesized sialic acids offers insights into their biological function.



The incorporation of sialic acids into macromolecules modulates numerous cellular and molecular interactions, including many that are involved in nervous system development and function.[1] It was recently shown that *Drosophila melanogaster* (*Dm*) have N-linked glycans containing sialic acids.[2,3] Based on sequence homology with their counterparts in vertebrates, various enzymes in the sialylation pathway have been cloned from *Dm*. The sialic acid synthase (*Dm*SAS) was found to generate phosphorylated forms of sialic acids when expressed in bacteria and in insect Sf9 cells (derived from *Spodoptera frugiperda*).[4] The CMP-sialic acid synthase (*Dm*CSAS) was found to generate CMP-sialic acids when expressed in mammalian cells.[5] The α2−6-sialyltransferase (*Dm*SialT), however, is the only *Dm* sialylation pathway enzyme shown to be functional in live *Dm* S2 cells.[6] These studies indicate that the sialylation pathway of *Dm* appears to be intact and similar to that of vertebrates.[7] Recently it was shown that a mutation in the *Dm*SialT, which resulted in the loss of *Dm*SialT activity *in vitro*, affected various *Dm* functions *in vivo*, indicating that sialylation is important for normal function in *Dm*.[8] Similar findings have been observed with a mutation in the *Dm*SAS (Akan and Palter, unpublished). These reports indicate that the function of these various enzymes is important in *Dm*. Moreover, on the basis of their characterization in expression systems, these enzymes are likely involved in the sialylation pathway.

The investigation of sialylation in insect cells has been limited by a lack of facile labeling tools. Lectins, for example, have not proved adequate for the detection of the low levels of sialic acids found in *Drosophila* due to the generally low binding affinity and poor specificity of these probes. This project exploits bioorthogonal labeling techniques developed by the metabolic glycoengineering field[9] to stably label newly synthesized sialo-glycoconjugates

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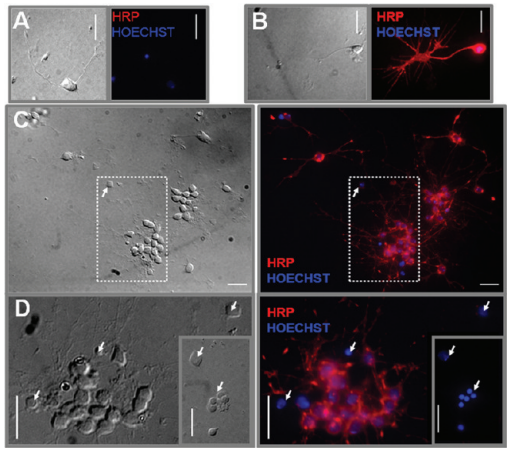

Figure 1. Dissociated CNS cells displayed neuritic outgrowth and were horseradish peroxidase (HRP)-positive. Dissociated central nervous system (CNS) cultures (2 CNS/corverslip) were incubated for 1 day and immunostained with anti-HRP, and the nuclei were stained with Hoechst. Left panels show the corresponding light picture. (A) Negative control for anti-HRP (exposed only to the secondary antibody: Alexa-Fluor-546-Goat-Anti-Rabbit). (B−D) Positive labeling with anti-HRP. The main image in panel D corresponds to the inset area in panel C that was rotated by 90°. The inset in \panel D corresponds to another field that shows a group of HRP-negative cells. The arrows indicate some of the HRP-negative cells. (63x objective, bar = 10 μm).

with covalently attached fluorophores. By using this approach with azido-modified *N*-acetymannosamine (ManNAc) analogues that serve as selective metabolic precursors for sialic acid biosynthesis,[10] we used a commercial adaptation of Click-iT chemistry to label and detect sialic acids in cultured CNS neurons. We found that CNS neurons isolated from the wild type *Dm*, but not those isolated from the *Dm*SAS mutant, incorporate sialic acids. The results support the view that endogenous *Dm*SAS is functional in *Dm* neurons and that its function is essential for sialic acid incorporation.

RESULTS AND DISCUSSION

Neuronal Cultures. The CNS cultures from *Dm* third instar larvae contained the previously described cell types[11] (Supplemental Figure S1). To assess the proportion of neurons in these cultures, cells were stained with anti-horseradish peroxidase (anti-HRP) a neuronal marker in *Drosophila*.[12] Most of the cells were HRP-positive and displayed neurites (Figure 1). There was a small population of HRP-negative cells that did not have neurites and could be found individually and within clumps (Figure 1D).

***Dm*SAS and Sialic Acid Incorporation.** To assess whether *Dm* CNS neurons incorporate sialic acids into glycoconjugates, cultures were incubated with 30−50 μM sialic acid precursor peracetylated-*N*-azido-mannosamine (Ac_4ManNAz or NAZ). The cellular biosynthetic utilization of NAZ was detected using the Click-iT assay to label Sia5NAz (a sialic acid form) that had been incorporated into cellular glycoconjugates, as described under Methods and schematically represented in Figure 2A,B. As controls, cultures were incubated with 30−50 μM nonazide, natural sialic acid precursor peracetylated-*N*-acetyl-mannosamine (Ac_4ManNAc or NAC). Figure 2 shows pictures of neurons from the wild type (w^{1118}) (Figure 2C,D) and from the *Dm*SAS mutant (2d/2d) (Figure 2E) stained with anti-HRP and labeled for Sia5NAz incorporation. Only the somas displaying fluorescence levels that were above that displayed by the somas in the NAC group were considered to have detectable levels of Sia5NAz. It was found that an average of 22% of the w^{1118} neurons displayed detectable levels of Sia5NAz incorporation, whereas none of the neurons from the *Dm*SAS mutant (2d/2d) displayed detectable levels of Sia5NAz incorporation (Figure 2F). Moreover, reintroduction of *Dm*SAS to 2d/2d, that is the 4−4 rescue mutant, allowed Sia5NAz incorporation of an average of 31% of the neurons (Figure 2F). The *Dm*SAS gene was reintroduced using a heat shock promoter, and hence it is expressed in all cells. These results indicate that the *Dm*SAS mutation was responsible for blocking incorporation of sialic acids.

Serum Helps Sialic Acid Incorporation. The number of neurons labeled for Sia5NAz incorporation was highly reduced when the cells were cultured in serum-free medium (SFM) (Figure 2G). On average only ∼2% of the w^{1118} and 4−4 neurons cultured in SFM in the presence of 30−50 μM NAZ displayed detectable levels of Sia5NAz incorporation (Figure 2G). This percentage was significantly lower compared to that observed when neurons were cultured with the same level of NAZ in serum-containing medium (Figure 2F). By heat inactivating the serum, we sought to determine if the serum contained an active protein that assisted in either NAZ uptake or processing. We found that cultures containing heat-inactivated serum and either 30 μM (not shown) or 250 μM NAZ showed no reduction in Sia5NAz incorporation as compared to cultures containing nonheat-inactivated serum (*e.g.*, Figure 3). We have not yet identified the serum component(s) that facilitate the incorporation of sialic acids. However, this effect does not appear to involve an active protein since it was observed even when CNS neurons were cultured in medium containing heat-inactivated serum. Although we have not rigorously ruled out the possibility, the serum effect also does not appear to involve attachment factors since the attachment of CNS neurons appear to be comparable in both SFM and serum-containing media. Under SFM, however, increasing the levels of NAZ from 30 to 50 μM to 250 μM increased the percentage neurons incorporating Sia5NAz from 2% to ∼31% in 2- to 3-day cultures. Hence, the presence of serum is not required, but it facilitates the incorporation of Sia5NAz.

Sialic Acid Incorporation in Neurites. To determine if neurites incorporated sialic acids, cells were incubated with a high level of NAZ (250 μM) in the presence of serum. Neurites were visualized through HRP labeling (Figure 3A,B). Under these conditions all of the w^{1118} (Figure 3A−D) and 4−4 (Figure 3E,F) neurons (cells with neurites) incorporated Sia5-NAz in the soma and, to a lower extent, in their neurites. Within the neurites, the level of Sia5NAz incorporation was higher in the proximal than in the medial and distal regions (Figure 3D,F). Many of the cells without neurites (round cells) were HRP-positive and also incorporated Sia5NAz (Figure 3A). Interestingly, some of the round HRP-negative cells also incorporated Sia5NAz, but to a lower extent than the HRP-positive cells (Figure 3C,E). Under the same conditions, none of the cells in 2d/2d cultures displayed Sia5NAz incorporation (not shown). Since neuronal precursors have been shown to be HRP-positive,[13] then some of the round cells could be neuronal precursors or neurons that failed to extend neurite outgrowth.

Giant Neurons Incorporate Sialic Acids. Giant neurons are part of the giant fiber system that in many invertebrates mediate the "escape" or "startle" response.[14] Transcripts of SialT have been reported in a subset of CNS neurons in third instar larvae,

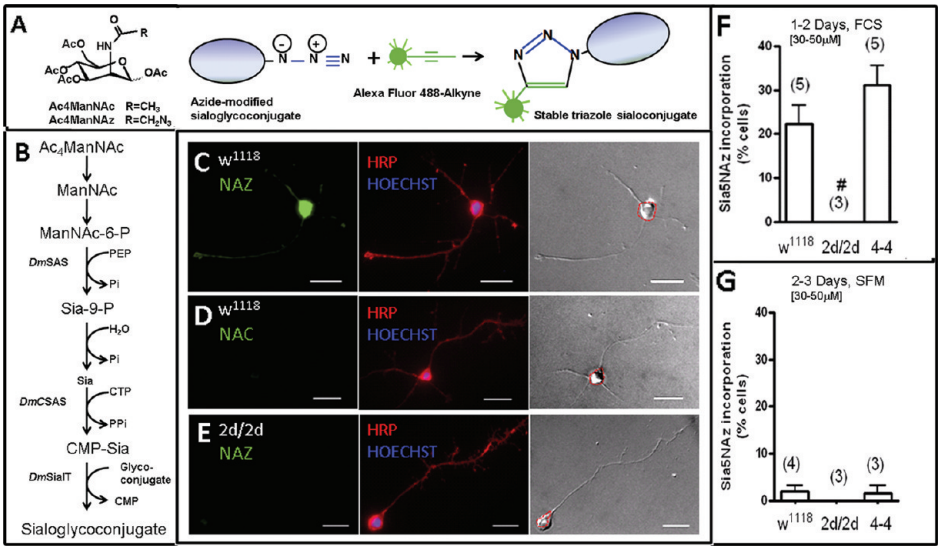

Figure 2. Cultured CNS neurons from w^{1118} and 4−4, but not those from 2d/2d, incorporate sialic acids. (A) Molecular structure of Ac_4ManNAc (NAC) and Ac_4ManNAz (NAZ) and a schematic representation of the Click-iT reaction between the azide-modified sialoglycoconjugates and alkyne group on the fluorescent agent. (B) General pathway for sialic acid incorporation into glycoconjugates in *Dm*. Once in the cytoplasm nonspecific esterases remove the acetyl groups in NAC and NAZ, converting them into ManNAc and ManNAz. ManNAc (or ManNAz) is processed to become CMP-Sia (cytidine monophosphate-sialic acid) (or CMP-Sia5NAz) which is the sialic acid donor molecule. *Dm*SAS: *Dm* sialic acid synthetase; *Dm*CSAS: *Dm* CMP sialic acid synthetase; *Dm*SialT: *Dm* sialyltransferase; PEP: phosphoenolpyruvate; Sia-9-P: sialic acid 9-phosphate; Pi: phosphate; CTP: cytidine triphosphate; PPi: pyrophosphate. (C−G) Dissociated CNS cultures (1−3 CNS/coverslip) were cultured in either serum-containing medium (C−F) or in SFM (G) for 1−3 days in the presence of NAZ or NAC (30−50 μM). (C−E) Pictures of neurons that were immunostained with anti-HRP and the nuclei stained with Hoechst (middle panels). Staining with the Alexa-Fluor-488-Alkyne (left panels). The red dotted lines outline the soma (light pictures, right panels). Incorporation of Sia5NAz (fluorescent intensity labeling) was measured in individual neuronal somas. (63x objective, bar = 10 μm). (F) Mean percentage of neurons that incorporated Sia5NAz when cultured in serum-containing medium; $n = 3-5$ separate experiments. One-way analysis of variance ($P < 0.001$); post test Bonferroni, #$P < 0.05$ between the 2d/2d group and the w^{1118} group. No difference was found between the 4−4 group and the w^{1118} group. Sia5NAz incorporation was measured in 25−100 neurons per condition in one experiment and in 102−150 neurons per condition in four experiments. (G) Mean percentage of neurons that incorporated Sia5NAz when cultured in SFM; $n = 3-4$ separate experiments. No significant difference was found between any of the groups (One-way analysis of variance). Sia5NAz incorporation was measured in 103−146 neurons per condition in the four experiments.

particularly where giant neurons are located.[7] However, expression of SialT was shown to be present in a larger number of neurons, but not in glia, throughout the third-instar larval CNS.[8] Expression of SialT was highest in the region where giant neurons are located.[8] Hence we decided to also measure sialic acid incorporation in the giant neurons. We first used *Dm* in which their giant neurons were labeled with GFP (membrane-targeted green fluorescent protein) to determine whether we could identify giant neurons in the dissociated CNS cultures solely on the basis of their morphology/size. We confirmed that GFP labeling was detected within the CNS region where giant neurons are located (Figure 4A) and found that the giant neurons could easily be identified from the rest of the cultured cells solely on the basis of their size (diameter ≥20 μm) (Figure 4B). Hence we identified giant neurons in CNS cultures of our experimental flies solely on the basis of their size. Most of the giant neurons did not survive in culture. Two days after dissociation, some of them attached, and their diameters were more than double that of nonattached giant neurons (Figure 4C−F). Although the culture conditions are not optimal for giant neurons (indicated by the low level of HRP-staining and their short survival time of 1−2 days), the giant neurons incorporated Sia5NAz to a higher extent than the smaller CNS neurons (Figure 4G). This finding is consistent with previous observations where SialT expression was reported to be highest in third instar larvae brain regions where giant neurons are located.[7,8]

Sialic Acids in the Plasma Membrane. Visual inspection shows, as expected, strong HRP labeling at the plasma membrane, whereas Sia5NAz labeling occurs throughout the cell (Figure 3B). In some neurons, Sia5NAz labeling appears to be highest at the perinuclear region (Figure 3B). To confirm that sialo-glycoconjugates are also located at the plasma membrane, we carried out Sia5NAz labeling in live cells. In live cells, labeling of Sia5NAz was limited to the cell surface and displayed high co-localization with HRP labeling (Figure 5A). Detectable levels of Sia5NAz incorporation in the plasma membrane were observed in an average of 30% of the w^{1118} neurons, in none of the *Dm*SAS mutant (2d/2d) neurons, and in 33% of the 4−4 rescue mutant neurons (Figure 5C). These results indicate that sialo-glycoconjugates are present in the plasma membrane of *Dm* CNS neurons.

Sialidase Treatment. To confirm that NAZ was converted to Sia5NAz and eventually incorporated into glycoconjugates, we treated live CNS neurons with sialidase prior to carrying out Sia5NAz labeling. Figure 5 shows images of w^{1118} CNS neurons that were cultured with NAZ for 6 days and then either not treated (Figure 5D) or treated with sialidase (Figure 5E). Sialidase treatment completely eliminated the Sia5NAz labeling in w^{1118} and 4−4 CNS neurons (Figure 5F). These results support the view that the labeling observed in NAZ-treated cells represents the actual incorporation of Sia5NAz into sialylated glycoconjugates.

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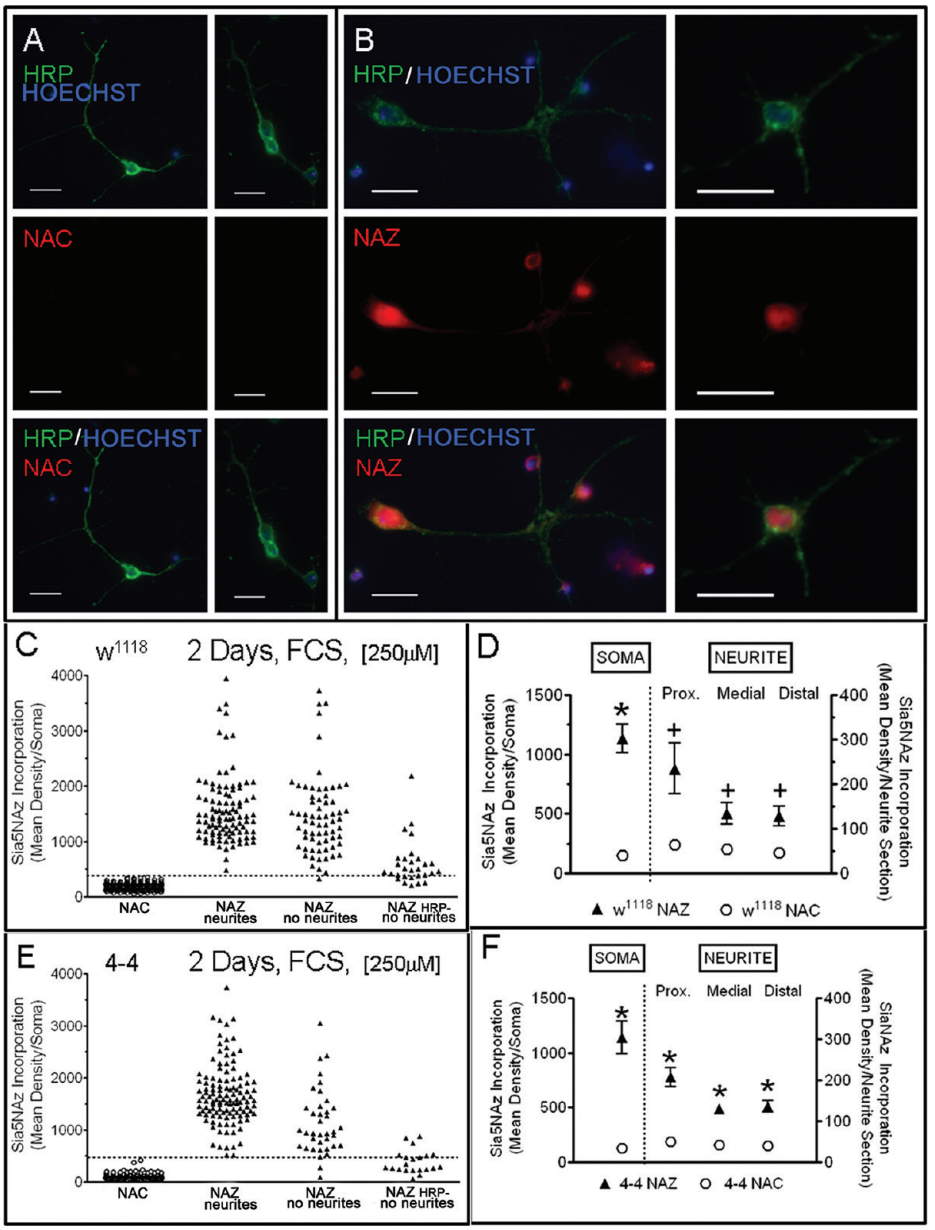

Figure 3. Incorporation of Sia5NAz into the soma and neurites of CNS cells. Cells were cultured for 2 days in medium containing heat-inactivated serum and 250 μM of NAC or NAZ (5 CNS/coverslip). Dissociated neurons from w^{1118} (A, B) and 4−4 (not shown), immunostained with anti-HRP and stained with the Alexa-Fluor 546 Alkyne and Hoechst for nuclei. (63x objective, bar = 10 μm). (C, E) Fluorescent intensity labeling for all of the cells. For the NAZ group the cells were separated into 3 groups: cells with neurites and that were HRP-positive ("with neurites"), cells that have no neurites and were HRP-positive ("no neurites"), and cells that have no neurites and were HRP-negative ("no neurites HRP−"). The total number of cells for w^{1118} were 226 (NAC), 101 (NAZ, "with neurites"), 67 (NAZ, "no neurites"), and 29 (NAZ, "no neurites HRP−"). For 4−4 the numbers were 279 (NAC), 118 (NAZ, "with neurites"), 39 (NAZ, "no neurites"), and 21 (NAZ, "no neurites HRP−"). (D, F) Fluorescent intensity labeling in the neurites was measured in 20 randomly selected neurons in the NAZ and NAC groups from w^{1118} (D) and 4−4 (F) cultures. We selected neurons that had neurites that were longer than 5 times their diameter and in which we could identify the location of the neurite terminal. If the neuron had more than one neurite, we selected the predominant one. Measurements were done in three regions (10 μm in length): proximal (Prox.), close to the soma; distal, close to the neurite terminal but prior to any terminal branches; and medial, halfway between the proximal and distal regions. Unpaired *t*-test, two-tailed; $^*P < 0.0001$; $+P < 0.01$, group with respect to the NAC group.

***Dm*SialT Expression in Cultured Neurons.** In Western blots the anti-*Dm*SialT detected a strong band at the molecular weight expected for *Dm*SialT only in HEK293 (human embryonic kidney) cells that were transfected with the *Dm*SialT (Figure 6A). Moreover, immunostaining with anti-*Dm*SialT gave a positive signal only in HEK293 cells that were transfected with the *Dm*SialT (Figure 6B). For immunostaining of CNS neurons we used three negative controls, in which the primary antibody was replaced with nothing, preimmune serum, or purified rabbit IgG. The background level was comparable for the three negative controls (Figure 6C−G). Although the level of expression differed, essentially all of the neurons from w^{1118} (Figure 6H−J) and

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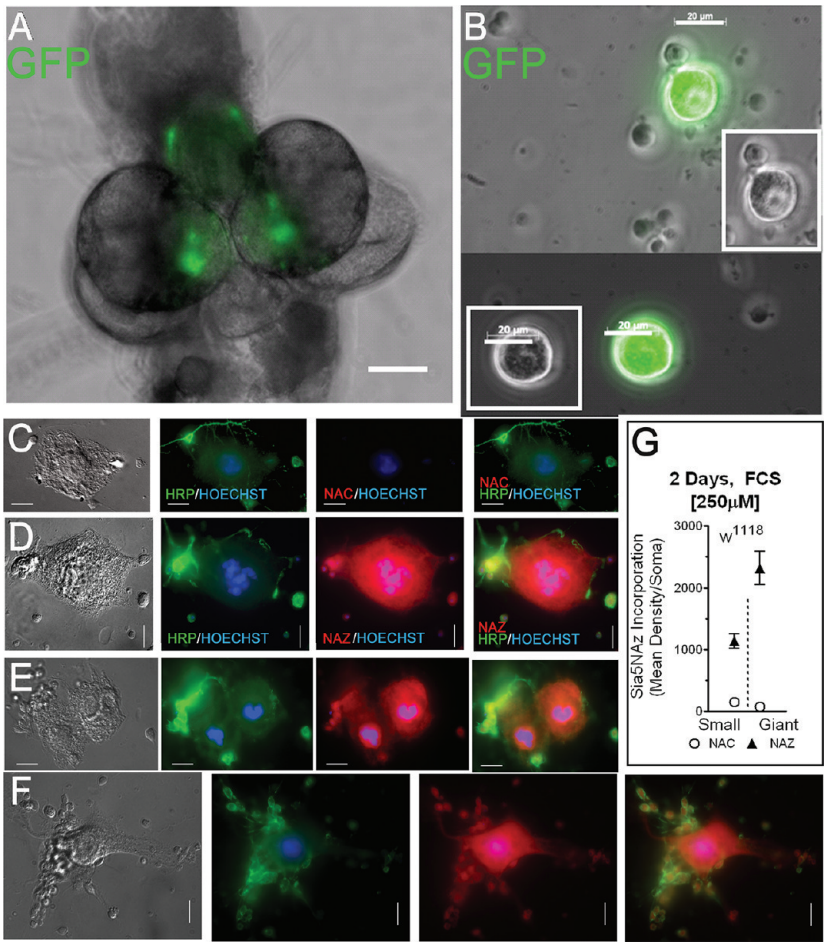

Figure 4. Cultured giant neurons incorporate sialic acids. (A, B) Identification of giant neurons in third instar larvae in whole CNS (10x objective, bar = 100 μm) (A) and dissociated CNS (40x objective, bar = 20 μm) (B). The giant neurons were labeled with membrane-targeted green fluorescent protein (GFP) by using UAS-mc8-GFP driven by OK307-Gal4. (C−F) Pictures of giant and smaller CNS neurons derived from w^{1118} *Dm*. Cells (5 CNS/coverslip) were cultured for 2 days in medium containing heat-inactivated serum and 250 μM of either NAC (C) or NAZ (D−F). Cells were immunostained with anti-HRP and stained with the Alexa-Fluor-546-Alkyne and Hoechst for nuclei. (63x objective, bar = 10 μm). (G) Quantification of Sia5NAz incorporation in giant neurons (n = 7) as compared to that of close-by smaller neurons (n = 34) (mean ± sem).

2d/2d (Figure 6K−M) expressed SialT. These data also indicate that the expression of SialT was not modified in the 2d/2d mutant.

Our data show that in culture a large proportion of the *Dm* CNS neurons express SialT and are capable of incorporating Sia5NAz, a somewhat surprising finding considering that previously SialT transcript was detected only in a small number of CNS neurons.[6−8] However, another study found that the proportion of CNS neurons in the third instar larvae that express SialT is much larger although not 100%.[8] One explanation for the nearly 100% of cells with neurites found to stain for SialT is that since insect cells release SialT,[6] the released SialT was possibly taken up by neighboring CNS neurons. Another possibility is that the neuronal populations *in vivo* are not equally represented in culture. Supporting this view is the observation that after 1 day in culture most of the giant neurons and some other cells are lost, whereas neuroblast-like cells continuously undergo asymmetrical division, giving rise to new neurons. Another possibility is that the SialT expression in *Dm* CNS neurons is enhanced in culture as a result of environmental changes, as it has been shown in cultured mammalian cells.[15] Finally, it is also possible that the proportion of neurons that incorporate sialic acids *in vivo* may actually be higher than previously described and that in whole tissue only the fraction of cells expressing the highest enzymatic levels can be easily detected. Our data show that giant neurons appear to incorporate significantly larger amounts of Sia5NAz than other smaller CNS neurons (Figure 4). This observation is consistent with previous findings that SialT is predominantly, but not solely, detected in third instar larvae CNS regions where the giant neurons are located.[7,8]

Implications. Our finding that *Dm*SAS activity is required for sialic acid incorporation in CNS neurons suggests that the lack of sialylation in CNS neurons contributes to the observed behavioral changes of the *Dm*SAS mutant (Akan and Palter, unpublished). Recently, it was reported that the *Dm*SialT mutant also displays behavioral changes; this mutant has a lower number of muscle synaptic branches and boutons and displays smaller evoked excitatory junction potentials and sodium (Na^+) currents with lower tetrodotoxin (TTX) sensitivity.[8] The correlation between the loss of SialT activity of the mutated *Dm*SialT *in vitro* and the functional changes *in vivo* strongly supports the notion that sialylation is important for normal function in *Dm*.[8] Changes in TTX sensitivity have been shown to be due to changes in the amino acid sequence of Na^+ channels;[16] hence the change in TTX-sensitivity of Na^+ currents in the SialT mutant could reflect

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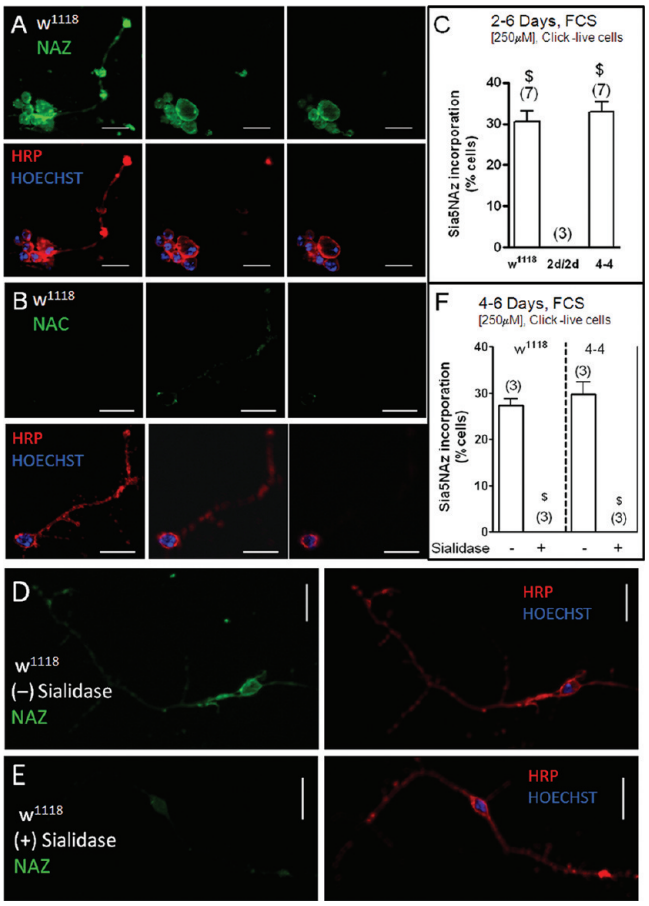

Figure 5. Cultured CNS neurons from w^{1118} and 4−4, but not those from 2d/2d, incorporate sialo-glycoconjugates in the plasma membrane that were sensitive to sialidase treatment. Cells were cultured for 2−6 days in serum-containing medium and 250 μM NAZ (or NAC) (1−3 CNS/coverslip). The Click-iT reaction with Alexa-Fluor-488-Alkyne was carried out in live cells; the immunostaining with anti-HRP and nuclear labeling with Hoechst were done following fixation. (A, B) Pictures of neurons taken using the Apotome Zeiss system, (63x objective, bar = 10 μm), panels going from left to right show pictures of 1 μm thickness taken from the bottom toward the top. (C) Mean percentage of neurons that incorporated Sia5NAz in the plasma membrane. Sia5NAz incorporation was measured in 62−121 neurons per condition in each experiment. (n = no. of experiments). One-way analysis of variance ($P < 0.0001$); post test Bonferroni, $\$P < 0.001$ between the group and the 2d/2d group. No significance difference was found between the w^{1118} and the 4−4 groups. (D, E) Pictures taken with the Apotome Zeiss system (1 μm thickness, 63x objective, bar = 10 μm) of untreated and sialidase treated cells. (F) Mean percentage of w^{1118} and 4−4 neurons that incorporated Sia5NAz in the plasma membrane in untreated and sialidase treated cultures. Sia5NAz incorporation was measured in 78−121 neurons per condition in each experiment. $n = 3$ separate experiments. One-way analysis of variance ($P < 0.0001$); post test Bonferroni, $\$P < 0.001$ between the sialidase treated and untreated group. No significance difference was found between the untreated w^{1118} and the 4−4 groups.

changes in the expression or trafficking of different Na^+ channels. In fact, the trafficking of Na^+ channels[17] and of some K^+ channels[18,19] have been shown to be affected by glycosylation.

In vertebrates, sialylation has been shown to affect the function of some channels including Na^+[20−22] and some K^+[18,23] channels. When expressed in mammalian cells, sialylation also affects the function of *Dm* shaker-K^+ channels.[24] In addition, sialylation of membrane lipids can also modify the function of Na^+ channels.[25] Hence, alterations of electrophysiological measurements in SialT mutant can also involve functional changes in both Na^+ and K^+ channels. Our demonstration that some of the sialo-glycoconjugates are located at the plasma membrane indicates that these molecules have the correct spatial localization to be Na^+ and K^+ channels and thus suggests that changes in sialylation in *Dm* neurons could affect their excitability. In summary, this is the first study that shows that an endogenously expressed *Dm* enzyme is functional in the sialylation pathway. We further found that *Dm*SAS is functional in most, if not all, cultured CNS neurons from third instar larvae and that *Dm*SAS is required for the incorporation of sialic acids in CNS neurons.

METHODS

Neuronal Cultures. The third-instar larva is one of the stages in the developmental cycle of *Dm*. The eggs hatch and give rise to first instar larvae. The larvae grow while molting twice into second and third instar larvae. Then the larvae encapsulate in the puparium and undergo metamorphosis after which the adult flies emerge.[26] CNS neuronal cultures from third instar larvae were prepared by modifying a previously reported protocol.[11] Third instar larvae were placed into a well (staining glass plate) containing ~300 μL of 70% (v/v) ethanol for 2 min. Larvae were rinsed by transferring them consecutively through three wells containing phosphate-buffered saline (PBS). While holding the body with a forceps placed at about 1/3 of the body length from the mouth, the mouth part was held with a second forceps and pulled away from the body. The mouth parts with the attached CNS (brain hemispheres and ventral nerve cord) were moved into a new well containing PBS, and the CNS was isolated under a dissecting microscope. Dissociation was done by placing the pooled CNS into a 1.5 mL conical eppendorf tube containing 0.025% (w/v) of trypsin and incubating for 15 min at room temperature (RT). Afterward, the tube was placed in a hybridization incubator rotating shaker (Robbins Scientific, model 1000) at full speed (20 rpm) for 15 min at 37 °C. Then, 500 μL of the culture media with or without 10% (v/v) fetal calf serum (FCS) was added. The culture media consisted of Scheneider's Drosophila Medium (Gibco) supplemented with 2% (v/v) B-27 Serum-Free Supplement (GIBCO), 50 μg mL^{-1} insulin, 100 μg mL^{-1} transferrin; penicillin (100 U mL^{-1}) and streptomycin (100 μg mL^{-1}) (Sigma). The tissue was dissociated by first triturating 40 times with a 1 mL micropipet and then 20 times with a fired polished glass Pasteur pipet. Clumps were allowed to settle down, and the supernatant was removed and collected in a 15 mL conical tube. More culture media (~1 mL) was added to the clumps and the tissue was triturated again 20 times. The last two steps were repeated three to five times. The pooled supernatants were spun for 5 min in a tissue culture centrifuge (Beckman JS 4.2: 2510g). The pellet was resuspended by adding fresh medium (~20 μL per 2−5 brains; ~7200 cells/CNS). Then 20 μL of cell suspension was placed in the middle of each poly-D-lysine precoated (12 μg mL^{-1} for 5 min) round coverslip (12 mm diameter), which were placed in 4 well culture dishes. Cells were allowed to attach for 20 min while in the incubator (humidified air, 25 °C). Following cell attachment, 800 μL of culture medium was added to each coverslip. The medium was changed every other day.

Click-iT Reaction. Sialo-glycoconjugates that have incorporated Sia5NAz can be detected by the "Click-iT" commercial adaptation (Invitrogen Molecular Probes; for fixed cells catalog no. C10269; for live cells catalog no. C10405) of fluorescent labeling strategies reported by Wong's laboratory that exploit the Click-iT reaction between azide and alkyne groups.[27] Control cells were grown in the absence of NAZ or in the presence of NAC.[28] The NAC analogue reproduces the increased

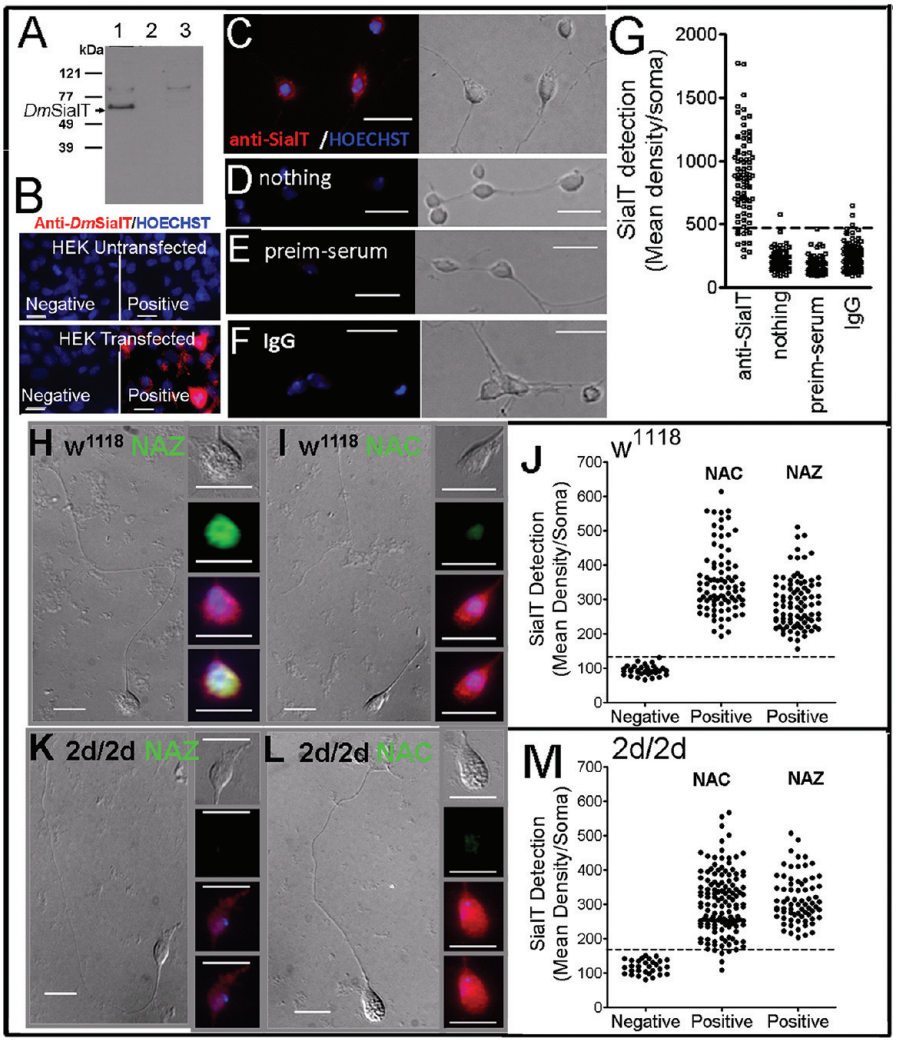

Figure 6. Most of the CNS neurons express SialT. (A) Western blot for the *Dm*SialT (*Dm* α2−6-sialyltransferase). The arrow indicates the location of *Dm*SialT. The locations of the molecular weights ladder are indicated. Lane 1: HEK293 (human embryonic kidney) cells transfected with *Dm*SialT; Lane 2: loading buffer; lane 3: untransfected HEK293 cells. For each lane 50 μg of protein was mixed with 5XSDS loading buffer, heated to 80 °C for 20 min, and then loaded onto 8% (w/v) SDS-PAGE. The blot was probed overnight at 4 °C with anti-*Dm*SialT (1:1000 dilution). (B) Untransfected (top) and transfected (bottom) HEK293 cells with *Dm*SialT. Cells were immunostained with anti-*Dm*SialT (1:10,000 dilution) and the nuclei stained with Hoechst (63x objective, bar = 20 μm). (A, B) HEK293 cells were transfected with 1 μg of pCDNA3.1 *Dm*SialT plasmid by using lipofactamine (Invitrogen). (C−G) Dissociated *Dm* CNS cells immunostaining with anti-*Dm*SialT. Cells were cultured for 1 day in serum-containing medium (2−3 CNS/coverslip). (C) Cells were first incubated with the anti-*Dm*SialT (1:10,000 dilution, 0.1 μg mL^{-1}) and then with the secondary antibody Alexa-Fluor-546-Goat-Anti-Rabbit. (D−F) Three negative controls were done, in which the primary antibody was replaced by either (D) nothing, (E) preimmune serum (0.05 μL preimmune serum mL^{-1}, ~0.1 μg IgG mL^{-1}), or (F) purified rabbit IgG (0.1 μg mL^{-1}). The secondary antibody Alexa-Fluor-546-Goat-Anti-Rabbit was added in all cases. The nuclei were stained with Hoechst. The corresponding light pictures are shown (63x objective, bar = 10 μm). (G) Quantification for SialT intensity labeling was done by measuring the fluorescent intensity labeling per each soma (each dot represent a soma). The background level was given by the three negative controls (dashed line). Most of the neurons displayed positive labeling for SialT. Pictures of dissociated neurons from w^{1118} (H, I) and 2d/2d (K, L) were immunostained with anti-*Dm*SialT and stained with the Alexa-Fluor-488-Alkyne and Hoechst for nuclei. (63x objective, bar = 10 μm). Cells were cultured for 7 days in SFM. Most of the neurons from w^{1118} (J) and from 2d/2d (M) were positive for anti-*Dm*SialT.

flux through the sialic acid pathway supported by NAZ and is used to control for potential dose limiting toxicity of these analogues.[28,29]

NAC and NAZ were synthesized as previously described,[30,31] and the stock solutions of (25 mM) were prepared in 95% (v/v) ethanol. The Click-iT reaction was carried out following the manufacture instructions. For fixed cells, the attached cells were washed once with PBS and then fixed with 4% (w/v) paraformaldehyde (pH = 7.4; 15 min, RT). Coverslips were rinsed three times with PBS at RT. The cells were permeabilized by incubating with 0.5% (v/v) Triton X-100 in PBS (15 min, RT). Coverslips were rinsed three times with PBS at RT and left in 1% (w/v) BSA (or 10% (v/v) goat serum when immunostaining will follow) for 1 h. Under dark conditions the reaction-cocktail containing the Alexa-fluor-488-Alkyne or the Alexa-fluor-546-Alkyne (2.4 μM) was added. The samples were rinsed three times with PBS. The nuclei were counter stained with Hoechst 33342 (20 μg mL^{-1}, 10 min, RT), and coverslips were mounted into slides using 1 drop of Aqua Poly/Mount (Polysciences, Inc.). In the initial experiments the cells were rinsed with PBS containing 1% (w/v) BSA.

***Drosophila* Stocks.** A deletion of *Dm*SAS was created by imprecise excision of a P element; in the selected line (2d) the P element was deleted as well as 480 bp of flanking DNA including 148 bp of a highly

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conserved coding region of *Dm*SAS (Akan and Palter, unpublished). The 4−4 rescue line carries a cDNA for *Dm*SAS on both second chromosomes (Akan and Palter, unpublished). The w^{1118} was used as the control fly strain as it is nearly isogenic with 2d/2d and is wild type for the sialic acid pathway enzymes. Third instar larvae heterozygous for the SAS deletion 2d/TM6Tb, produced a shortened body shape, tubby. Third instar larvae homozygous for the SAS deletion 2d/2d have normal body shape. To confirm the state of our fly stocks, we used single larva PCR. We also used PCR (on the pooled bodies parts left after removing the CNS) to confirm that the larvae from which the CNS tissue was isolated from were all homozygous for the *Dm*SAS mutation. PCR was done with the Extract-N-Amp Tissue PCR Kit (Sigma) following the manufacturer's instructions. DNA was extracted by first adding 10 μL of Extraction Solution and then 2.5 μL of Tissue Preparation Solution per fly. The mixture was incubated for 10 min at RT and then for 3 min at 95 °C . Finally 10 μL of Neutralization Solution B was added per fly and vortexed. A quick spin was done to pull the debris down. We used 4 μL of extract for the PCR reaction.

Drosophila melanogaster stocks UAS-mc8-GFP(3rd) and OK307-Gal4 were kindly provided by Dr. Greg Suh (NYU Langone Medical Center). Labeling (with membrane-targeted green fluorescent protein (GFP)) of giant neurons was done by using UAS-mc8-GFP[32,33] driven by OK307-Gal4.[34,35]

Immunocytochemistry. Cells attached to coverslips were rinsed 1 time with PBS, fixed (4% (w/v) paraformaldehyde, pH 7.4; 14 min), and rinsed 3 times with PBS at RT. Cells were permeabilized (0.5% (v/v) Triton X-100 in PBS, 15 min), rinsed 3 times with PBS, and incubated with a blocking solution (10% (v/v) goat serum in PBS, 1 h, RT). Then coverslips were incubated overnight (4 °C) with the primary antibody, rabbit anti-sialyltransferase (anti-*Dm*SialT; 1:10,000; final 0.10 μg mL^{-1}) or rabbit anti-horseradish peroxidase (anti-HRP; 1:20,000 Sigma-Aldrich no. P7899) in 2% (v/v) goat serum. After incubation with the primary antibody, coverslips were rinsed 3 times with PBS. Then coverslips were incubated (90 min, RT) with the secondary antibody Alexa-Fluor-546-Goat-Anti-Rabbit or Alexa-Fluor-488-Goat-Anti-Rabbit (1:1,500) and rinsed 3 times with PBS at RT. Anti-*Dm*SialT was raised using the peptide sequence CRFNHAPTQGHEVDVGSK-NH2 (243−260 aa). The peptide was conjugated to KLH and BSA and was produced by CPC Scientific, Inc. The rabbit IgG was from Jackson InmunoResearch. When the Click-iT reaction was combined with immunocytochemistry, the Click-iT reaction was done first.

Sialidase Treatment. The protocol previously used for cultured live mammalian cells[36] was adapted for cultured live drosophila CNS neurons. Cells were cultured in the presence of NAZ (250 μM) for 2−6 days in serum-containing medium. The serum-containing medium was removed, and the cells were washed once for 5 min with Scheneider's media. Then the Scheneider's media was replaced with fresh Scheneider's media with or without sialidase (0.005U mL^{-1}; neuraminidase from *Clostridium perfringens C. welchii*; Sigma) and incubated for 15 min at RT. The enzyme was removed by rinsing 3 times with PBS containing 0.1% (v/v) FCS (5 min/rinse, RT). Following the sialidase treatment the Click-iT reaction was carried out in live cells.

Image Acquisition. Images were captured (and analyzed) with a Zeiss Axiovert 200 (Germany) inverted microscope equipped with fluorescence and Nomarski optics, ApoTome (for optical sections), using an AxioCam camera and AxioVision Software version 4.6.3 (Zeiss). The filter set for detecting Alexa 488 (green): Excitation filter BP450−490; Beam Splitter FT 510; Emission BP 515−565. The filter set for detecting Alexa 546 (red): Excitation filter BP546/12; Bean Splitter FT 580; Emission filter LP 590. The filter set for detecting Hoechst (blue): Excitation filter G365; Bean Splitter FT 395 and Emission filter LP 420 or BP445/50. Images were taken from 20 to 30 random fields per coverslip. In each experiment they were two coverslips per treatment. The neuronal soma was outlined by hand.

■ ASSOCIATED CONTENT

Supporting Information. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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