Polysialic acid regulates sympathetic outflow by facilitating information transfer within the nucleus of the solitary tract


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Polysialic Acid Regulates Sympathetic Outflow by Facilitating Information Transfer within the Nucleus of the Solitary Tract

Expression of the large extracellular glycan, polysialic acid (polysia), is restricted in the adult, to brain regions exhibiting high levels of plasticity or remodeling, including the hippocampus, prefrontal cortex, and the nucleus of the solitary tract (NTS). The NTS, located in the dorsal brainstem, receives constant viscerosensory afferent traffic as well as input from central regions controlling sympathetic nerve activity, respiration, gastrointestinal functions, hormonal release, and behavior. Our aims were to determine the ultrastructural location of polysia in the NTS and the functional effects of enzymatic removal of polysia, both in vitro and in vivo. Polysia immunoreactivity was found throughout the adult rat NTS. Electron microscopy demonstrated polysia at sites that influence neurotransmission: the extracellular space, fine astrocytic processes, and neuronal terminals. Removing polysia from the NTS had functional consequences. Whole-cell electrophysiological recordings revealed altered intrinsic membrane properties, enhancing voltage-gated K^+ currents and increasing intracellular Ca^{2+}. Viscerosensory afferent processing was also disrupted, dampening low-frequency excitatory input and potentiating high-frequency sustained currents at second-order neurons. Removal of polysia in the NTS of anesthetized rats increased sympathetic nerve activity, whereas functionally related enzymes that do not alter polysia expression had little effect. These data indicate that polysia is required for the normal transmission of information through the NTS and that changes in its expression alter sympathetic outflow. Polysia is abundant in multiple but discrete brain regions, including sensory nuclei, in both the adult rat and human, where it may regulate neuronal function by mechanisms identified here.

Key words: electron microscopy; nucleus of the solitary tract; patch clamp; polysialic acid; sympathetic nerve activity; viscerosensory afferents

Significance Statement

All cells are coated in glycans (sugars) existing predominantly as glycolipids, proteoglycans, or glycoproteins formed by the most complex form of posttranslational modification, glycosylation. How these glycans influence brain function is only now beginning to be elucidated. The adult nucleus of the solitary tract has abundant polysialic acid (polysia) and is a major site of integration, receiving viscerosensory information which controls critical homeostatic functions. Our data reveal that polysia is a determinant of neuronal behavior and excitatory transmission in the nucleus of the solitary tract, regulating sympathetic nerve activity. Polysia is abundantly expressed at distinct brain sites in adults, including major sensory nuclei, suggesting that sensory transmission may also be influenced via mechanisms described here. These findings hint at the importance of elucidating how other glycans influence neural function.
Introduction

The extracellular space is complex, filled with molecularly diverse matrix, a dynamic structure that influences neuronal function (Venstrom and Reichardt, 1993; Theodosius et al., 2008). This extracellular matrix is enriched in proteoglycans, as well as membrane bound glycoproteins and glycolipids commonly terminated by sialic acids, that form elaborate coats around all cells (Schnaar et al., 2014).

Poly-sialic acid (polySia) is a long, homopolymer composed of eight or more α2,8-linked sialic acids (Schauer, 2009; Chen and Varl, 2010). It is linked predominantly to the neural cell adhesion molecule (NCAM) and its expression is developmentally regulated (Bonfanti et al., 1992; Rutishauser and Landmesser, 1996; Bonfanti, 2006; Hildebrandt and Dityatev, 2015). In these regions, polySia modulates synaptic plasticity and efficacy via a range of postulated mechanisms, including interactions with cell adhesion complexes, binding of neurotransmitter/neurotrophic substances, or via actions at glutamate receptors (Rutishauser and Landmesser, 1996; Rutishauser, 2008; Schnaar et al., 2014; Hildebrandt and Dityatev, 2015). In adults, polySia is also highly expressed in the nucleus of the solitary tract (NTS) (Bouzioukh et al., 2001a, b; Bonfanti, 2006). Despite such abundance, the precise location and function/s exerted by polySia in the NTS are unknown.

The NTS is a major integrative center receiving synaptic input from peripheral cardiovascular, respiratory, and gastrointestinal sensors as well as descending drive from higher brain centers, and is a critical relay of multiple homeostatic reflexes (Andresen and Paton, 2011; Pilowsky and Goodchild, 2002; Guyenet, 2006; Browning and Travagli, 2014). Glutamatergic viscerosensory afferents enter via the solitary tract and synapse with second-order NTS neurons (Talmann et al., 1980; Appleyard et al., 2007; McDougall et al., 2009; Jin et al., 2010). Information is then relayed within the NTS or transmitted to more distal nuclei (Aicher et al., 1995; Hermes et al., 2006), driving a range of autonomic, hormonal, and behavioral responses. Plasticity occurs within the NTS, providing a beneficial adaptive mechanism acutely but chronically can be maladaptive, resulting in cardiovascular, respiratory, or gastrointestinal dysfunction (Kline, 2008; Browning and Travagli, 2010, 2014; Zoccal et al., 2014). Such plasticity is mediated by changes in presynaptic or postsynaptic excitability, via mechanisms, including quantal size, vesicle turnover, or release probability (Kline, 2008). The contribution of glycans to neurotransmission and/or plasticity in the NTS is unknown.

Only one group has investigated polySia in the NTS, demonstrating that high-frequency stimulation of the solitary tract reduced polySia expression (Bouzioukh et al., 2001a, b). Intriguingly, similar stimulation paradigms modify the effectiveness of excitatory and inhibitory synapses within the area (Miles, 1986; Glaum and Brooks, 1996; Zhou et al., 1997), resulting in increased sympathetic drive (Sun and Guyenet, 1987). Together, these data suggest that polySia in the NTS could modify neuronal function and the transfer of information to downstream networks, altering sympathetic nerve activity.

In exploring this hypothesis, we verified the distribution of polySia immunoreactivity in the adult rat NTS and described its ultrastructural location. Next, we determined the intrinsic neuronal properties and signaling mechanisms in the NTS altered by enzymatic removal of polySia, using whole-cell recordings in brain slice. Finally, we examined the consequences of this removal on sympathetic nerve activity in vivo. polySia was present at sites regulating information transfer: in fine astrocytic processes, the extracellular space, and axon terminals. We used multiple enzymes (neuraminidases) to desialylate glycans, including the polySia-specific enzyme, endo-N-acetyl-neuraminidase F (endoNF) (Stummeyer et al., 2005). Enzymatic removal of polySia within the NTS altered passive membrane properties, perturbed both high- and low-frequency viscerosensory afferent input, and increased sympathetic nerve activity. Our data indicate that, within the NTS, polySia expression is crucial for maintaining neuronal function and afferent transmission which, if altered, impacts signaling at downstream networks modifying sympathetic outflow.

Materials and Methods

Animal welfare and ethical approval

All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were performed with the approval of the Macquarie University Ethics Committee (Animal Research Authorities: 2012/015, 2014/019, 2014/041). Animals were housed under constant 12 h light/dark cycles and allowed standard rat chow ad libitum.

Immunohistochemistry

Immunohistochemical procedures were performed as previously described (Parker et al., 2013; Bou Farah et al., 2016). Male Sprague Dawley rats (n = 4) were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and transcardially perfused with ice-cold DMEM followed by 4% PFA. Brains were removed and postfixed overnight in the same fixative. Coronal brainstem sections (40 μm) were cut using a vibratome (Leica VT 1200S). Free-floating sections were permeabilized in 50% ethanol for 30 min at room temperature and washed in Tris-PBS (TPBS, 10 mM Tris, 0.9% NaCl, 0.05% thimerosal) overnight at 4°C. Sections were incubated in primary antibodies (diluted in 10% normal horse serum in TPBS) against polySia (mouse Mab735, 0.1 μg/ml, RRID: AB_2619682) or synaptophysin (rabbit anti-synaptophysin, 1:500, Synaptic Systems, RRID: AB_2313584) and transcardially perfused with ice-cold DMEM followed by 4% PFA. Counterstaining was performed with fluorescent conjugated secondaries (Cy3-conjugated donkey anti-rabbit, 1:300, Jackson Immunoresearch Laboratories, #115-045-165, RRID: AB_2313584) for 2 h at room temperature. Sections were incubated in fluorescent conjugated secondaries (Cy3-conjugated donkey anti-rabbit IgG, 1:250, Jackson Immunoresearch Laboratories) for 2 h at room temperature. Sections were washed, mounted onto glass slides using Dako mounting medium, and visualized using a Zeiss upright microscope (Axio Imager Z.1). Images were acquired using ZEN 2012 imaging software (Zeiss). High-power confocal images were visualized using a Leica confocal microscope (Leica TCS SP8) and acquired using Leica Application Software AF (Leica). All images were imported and analyzed using the ImageJ plugin Fiji (Schindelin et al., 2012).
Electron microscopy

The method for electron microscopic immunocytochemistry was slightly modified (Llewellyn-Smith et al., 2005). Male Sprague Dawley rats (n = 4, 10–12 weeks of age) were anesthetized and perfused as described above with 4% formaldehyde and 0.3% glutaraldehyde (Electron Microscopy Sciences) in 0.1 M phosphate buffer, pH 7.4. Brains were removed and postfixed overnight in same fixative. Coronal sections of the medulla were cut into 50 µm sections using a vibrating microtome (Leica VT 1200S). Free floating sections were then permeabilized in 50% ethanol for 3 h and washed briefly in phosphate buffer. Sections were blocked using 10% normal horse serum in TPBS for 30 min and subsequently incubated in primary antibody against polycl (mouse Mab735, 1 µg/ml, RRID: AB_2619682) diluted in 10% normal horse serum in TPBS for 7 d. Sections were then incubated in biotinylated anti-mouse Ig (1:500, Jackson ImmunoResearch Laboratories, #715-065-150, RRID: AB_2307438) for 4 d followed by exposure to ExtrAvidin-Peroxidase (1:1500, Sigma-Aldrich, #E2886, RRID: AB_2620165) for a further 4 d. All steps were performed at room temperature with sections washed in TPBS (3 x 30 min) following each incubation. Immunoreactivity for polycl was detected with a nickel-intensified 3,3’-DAB reaction using glucose oxidase. Sections were reacted for 30 min (or until strong signal was detected), and the reaction was halted with three washes in TPBS.

After the DAB reaction, sections were washed in 0.1 M PBS, osmicated in 0.5% osmium tetroxide, stained en bloc with 1% aqueous uranyl acetate, and dehydrated through a graded series of acetone solutions. Sections were then infiltrated with 1:1 acetone:medium grade EPON resin and then in 100% resin overnight. Resin was periodically changed over 2 d before sections were flat embedded between glass slides and ACLAR plastic film and polymerized at 60°C for 48 h. Regions of interest dissected using a scalpel blade and mounted onto flat blank blocks. Ultrathin sections (60 nm) were cut using a diamond knife and an ultramicrotome (Ultracut, Leica). Ultrathin sections were collected onto 200 µm mesh copper grids. Selected grids were poststained with 2% uranyl acetate and Reynold’s lead citrate and imaged using a transmission electron microscope (JEM-1400, JOEL). Electron micrographs were acquired using Digital Micrograph Software (Gatan), imported to and analyzed using the ImageJ plugin Fiji (Schindelin et al., 2012).

In vitro electrophysiology data collection and analysis

Brainstem slice preparation

Coronal and horizontal brainstem slices were collected from male Sprague Dawley rats as described previously (Andersen and Yang, 1990; Titz and Keller, 1997). Coronal slices: Rats (weight: 50–250 g; mean age: P41 ± 2) were deeply anesthetized using isoflurane (5% in 100% O2, Cvent) and once nociceptive reflexes (hindpaw pinch) were absent, quickly decapitated. The brainstem was rapidly removed and placed in ice-cold aCSF (in mM as follows: 125 NaCl, 25 NaHCO3, 3 KCl, 1.25 NaH2PO4, H2O, 25 glucose, 2 CaCl2, 1 MgCl2, equilibrated with 95% O2–5% CO2). Coronal sections (300 µm) were cut using a vibrating microtome (Leica VT 1200S). Slices of the medial NTS (bregma: −13.92 to −13.56 mm) (Paxinos and Watson, 2006) were collected and placed in warm (34°C) aCSF and allowed to equilibrate for up to 60 min before recording. Horizontal slices: Rats (weight: 150–350 g; mean age: P56 ± 4) were deeply anesthetized using isoflurane (Cvent, 5% in 100% O2) and once nociceptive reflexes (hindpaw pinch) were absent, quickly decapitated. The brainstem was rapidly removed and placed in ice-cold aCSF. The cerebellum was then removed and a wedge of the ventral surface removed so as to orientate the brainstem at an angle that allows a 250-µm-thick slice to contain both the solitary tract and the nucleus. Slices were cut using a vibrating microtome (Leica VT 1200S) and placed in warm (34°C) aCSF. Slices were incubated for 60 min before recording. For “solitary tract evoked sustained current” protocol, the brainstem was initially incubated in ice-cold aCSF for 1 min, then sliced and incubated in Mg2+-free aCSF for 60 min at 32°C.

Recording parameters

Slices were placed in a custom-built chamber continuously bathed in aCSF warmed to 32°C (Temperature Controller TC-324B, Warner Instruments). Submerged sections were secured with a nylon harp and superfused at a rate of 2 ml/min. All recordings were made from neurons within the intermediate NTS. Neurons were visualized using an Olympus microscope (BX51WI). Patch recording electrodes (3.5–5.3 MΩ) were pulled using a P2000 laser pipette puller (Sutter Instruments) and filled with a potassium gluconate (K-Glu) internal solution consisting of (in mM) as follows: 10 NaCl, 130 K-Glu, 11 EGTA, 10 HEPES, 1 CaCl2, 2 MgCl2, 2 Na2ATP, 0.2 Na2GTP, 0.5% bicynchonin, pH 7.35–7.45, 290–295 mOsm. Recording electrodes were guided using a micromanipulator (Mp-225, Sutter Instruments).

Recordings were obtained using a Multiclamp 700B patch clamp amplifier (Molecular Devices), sampled at 20 kHz, and acquired using the 1401 CED computerized acquisition system (Cambridge Electronic Design), and analyzed offline using Spike2 software (Cambridge Electronic Design). The selection criteria for usable recordings were based on neuron stability, resting membrane potentials below −45 mV upon breaking in, series resistances <25 MΩ, and input resistances >200 MΩ. Series resistances were not compensated and liquid junction potentials not corrected. Data were not further analyzed if a ≥20% change in any of the parameters mentioned above was observed.

Steady-state outward currents

Coronal slices were initially incubated in aCSF, neuraminidase (Neu; 0.01, 0.1, 1 U/ml), or endoNF (0.1, 1 µg/ml) for 60 min at room temperature before recording. In whole-cell voltage clamp (−60 mV clamped potential), membrane potentials were stepped in 10 mV intervals to determine the currents generated (−70 to 30 mV, 100 ms step). aCSF and endoNF activated steady-state outward currents tested in the following paradigms: (1) in the recording pipette, EGTA was substituted with the calcium-chelating agent BAPTA (10 mM, Sigma-Aldrich #A9426); (2) the extracellular concentration of CaCl2 was lowered to 0.1 mM; and (3) before and after superfusion of tetraethylammonium (TEA, 10 mM; Sigma-Aldrich #T2265). Steady-state outward currents were measured as the last 50% of the current generated at each voltage step, normalized to baseline current. Two-way ANOVA with Bonferroni’s correction was used for data analysis of steady-state outward currents. Unpaired Mann–Whitney test was used for comparison between enzyme treatments.

Current evoked action potential discharge and after hyperpolarization (AHP). Coronal slices were initially incubated in either aCSF or endoNF (1 µg/ml) for 60 min at room temperature before recording. In current clamp (at resting membrane potentials), neurons were injected with depolarizing currents (10–100 pA, 100 ms per step) to evoke action potential discharge. The number of action potentials generated was then compared between endoNF and control (aCSF) treatment. The AHP was measured at the peak change in membrane potential (mV) 5 ms after termination of 100 pA current injection. AHP amplitude was then compared between endoNF and control (aCSF) treatment. Two-way ANOVA with Bonferroni correction was used for data analysis of action potential discharge. Unpaired t test was used for comparison.

Stimulus-evoked action potential generation

In horizontal slices, evoked action potential generation was investigated in previously identified monosynaptically connected neurons (described below). Action potential generation was initiated by solitary tract stimulation at 0.1 Hz, 20 stimuli delivered in a current-clamp mode. The number of action potentials generated in response to 20 stimuli (following frequency) was then compared before and 30 min after endoNF (1 µg/ml) infusion in the same neuron. The latency of action potential generation was calculated as the time between the stimulus artifact and the onset of the action potential. Paired t tests were used to compare responses.

Solitary tract evoked EPSCs and spontaneous EPSCs. A concentric bipolar stimulating electrode (200 µm outer tip diameter, Frederick Haer) was placed on the distal portion of the solitary tract rostral to the recording region. Using an Isolated Pulse stimulator (model 2100, A-M Systems), current shocks (100–800 µA, 50 µA) were delivered to the solitary tract every 6 s (shock duration 0.5 µs) until a compound evoked EPSC (eEPSC) was observed. The latency of eEPSCs was calculated as the time between the stimulus artifact and the onset of the action potential. Paired t tests were used to compare responses.

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Following a 30 min baseline period, injections were made with respect to recorded cells as described previously (Zhao et al., 2015). The sustained current was measured as the percentage change in current 100 ms following initiation of the first stimulus in the presence of Mg$^{2+}$-free aCSF alone and then following endoNF (1 μg/ml, 30 min) superfusion, in the same neuron. Paired t tests were used to compare responses.

**Sustained current records.** In slices incubated and recorded in Mg$^{2+}$-free aCSF, we first identified monosynaptically connected neurons as described above before delivering high-frequency current shocks (50 Hz, 5–10 stimuli) to the solitary tract to evoke NMDA-mediated sustained currents as previously described (Zhao et al., 2015). Following electrophysiological experimentation, slices were incubated for polySia immunoreactivity and recovery of lidocaine, 76.7 Na$_2$HPO$_4$, 26.6 NaH$_2$PO$_4$, 876 sucrose, 5 ethylene glycol, cryoprotectant consisting of the following (in mM): 0.5 polyvinylpyrrolidone, 76.7 Na$_2$HPO$_4$, 26.6 NaH$_2$PO$_4$, 876 sucrose, 5 ethylene glycol. Slices were then processed for polySia immunoreactivity and recovery of ligand, 76.7 Na$_2$HPO$_4$, 26.6 NaH$_2$PO$_4$, 876 sucrose, 5 ethylene glycol, cryoprotectant consisting of the following (in mM): 0.5 polyvinylpyrrolidone, 76.7 Na$_2$HPO$_4$, 26.6 NaH$_2$PO$_4$, 876 sucrose, 5 ethylene glycol.

Immunohistochemical characterization of recorded neurons

Following the electrophysiological experimentation, slices were incubated overnight in 4% PFA in PBS at 4°C followed by overnight incubation in cryoprotectant consisting of the following (in mM): 0.5 polyvinylpyrrolidone, 76.7 Na$_2$HPO$_4$, 26.6 NaH$_2$PO$_4$, 876 sucrose, 5 ethylene glycol. Slices were then processed for polySia immunoreactivity and recovery of recorded cells as described previously (Gogolla et al., 2006; Bou Farah et al., 2016). Briefly, free-floating sections were permeabilized using 0.5% Triton X-100 in 0.1% PBS, overnight at 4°C. Slices were then blocked in 5% BSA in PBS for 4 h at room temperature. The antibody against polySia (mouse Mab735, 0.1 μg/ml, RRID: AB_2619682) was added and slices incubated for 4 h at room temperature. Slices were then incubated in secondary antibody (Cy3-conjugated donkey anti-mouse IgG, 1:250, Jackson ImmunoResearch Laboratories #715-165-151, RRID: AB_2315777) and fluorophore-conjugated ExtrAvidin (ExtrAvidin-FITC, 1:500, Sigma-Aldrich, #E2761, RRID: AB_2492295) for detection of the biocytin-filled cells, overnight at 4°C. Slices were washed, mounted onto glass slides using Dako mounting medium, and visualized using either a Zeiss upright microscope (Axio Imager Z2), or Leica confocal microscope (Leica TCS SP8). Images were acquired and analyzed as described above.

In vivo electrophysiology data collection and analysis

**Surgery and electrophysiological recordings.** Male Sprague Dawley rats (n = 26, 10–12 weeks of age) were anesthetized with urethane (1.3 g/kg, i.p. 10% solution in saline, Sigma-Aldrich). Depth of anesthesia was assessed throughout each experiment by autonomic responses to hind-paw pinch. Core temperature was maintained between 36.5°C and 37°C by a homoeothermic heating blanket (Harvard Apparatus). The left femoral artery and vein were cannulated for measuring blood pressure and heart rate. Core temperature was maintained between 36.5°C and 37°C by a homoeothermic heating blanket (Harvard Apparatus). The left femoral artery and vein were cannulated for measuring blood pressure and heart rate.

**Tissue collection.** Western blot

In vitro tissue collection. Coronal brain slices were collected as described above. Slices were then incubated in either aCSF, neuramidase (0.1 U/ml, Sigma-Aldrich #N2876) or endoNF (1 μg/ml) (Stummeeyer et al., 2003) for 0, 45, and 60 min at room temperature. Slices were then trimmed by removing the ventral half of the slice below the hypoglossal nucleus and the dorsal region was stored and frozen at −80°C.

**Immunoblot.** In vivo tissue collection. To extract the brain region containing the NTS, brains were placed in an ice-cold brain matrix and a 2 mmrostrocaudal region of the brainstem was isolated as described previously (Damanhuri et al., 2012). The section was maintained frozen using dry ice and the region containing the NTS was hand cut using a scalpel blade (size 10).

**Electrophoresis.** Tissue was lysed using lysis buffer consisting of (in mM) as follows: 320 sucrose, 2 EDTA, 4 HEPES, and 1% SDS. Protein was extracted by incubating tissue on ice for 10 min, homogenizing with the FastPrep-24 homogenizer (MP Biomedicals) for 2 × 40 s cycles, and then centrifuged for 30 min at 13,200 rpm at 4°C. Supernatants were kept and protein concentrations determined using the BCA protein assay kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. Equal amounts of protein (20 μg) were loaded on a 7.5% polyacrylamide gel for electrophoresis. Proteins were then transferred onto a nitrocellulose membrane using the Trans-Blot Turbo System (Bio-Rad) and incubated in primary antibodies (diluted in 5% skim milk in PBS) against either polySia (mouse Mab735, 1 μg/ml, RRID: AB_2619682) or GAPDH (rabbit anti-GAPDH, I:5000, abcam, #ab9485, RRID:AB_307275) overnight at 4°C. HRP-secondary antibodies (goat anti-mouse IgG H and L, 1:10,000, and goat anti-rabbit IgG H and L, 1:10,000, R&D Systems, #HAF018, RRID: AB_573130, and #HAF008, RRID: AB_357235, respectively) were incubated on the membrane at room temperature for 2 h. Chemiluminescence detection was then performed using the Bio-Rad ECL kit and imaged using the Bio-Rad ChemiDoc system. All images were imported into ImageJ for densitometric analysis and expressed as a percentage change relative to protein loading control (GAPDH). One-way ANOVA with Bonferroni correction was used for comparison between treatments.

**Statistical analysis.** All values are expressed as mean ± SEM. Paired or unpaired t tests were used as indicated. One- or two-way ANOVA tests with Bonferroni post hoc tests were used as indicated. Mann–Whitney tests were used to determine whether different enzymes evoked different responses in sympathetic nerve activity. Statistically significant differences were considered at p < 0.05. All statistical analysis was performed using GraphPad Prism 6.

**Drugs, enzymes, and reagents.** All drugs, enzymes, and reagents, with the exception of endoNF (Stummeeyer et al., 2005) or TTX (Jomar Biosciences) were purchased from Sigma-Aldrich.
Results

PolySia is expressed abundantly within the neuropil of the NTS predominantly in fine astrocytic processes, the extracellular space, and apposing some synapses

In the adult brain, polySia is expressed abundantly within NTS, the adjacent area postrema, and dorsal motor nucleus of the vagus (Bonfanti et al., 1992). We confirmed these observations using the polySia specific antibody Mab735 (Frosch et al., 1985), demonstrating that a high density of expression was restricted to this, the dorsal vagal complex (Fig. 1A). polySia immunoreactivity (ir) was densely distributed throughout the intermediate NTS (Fig. 1B), as individual puncta or as punctate rings surrounding unlabeled soma (Fig. 1Bi, Bii). Similar expression patterns were evident throughout the rostrocaudal extent of the nucleus (data not shown), suggesting potential influence over all functions controlled by the NTS: respiratory (caudal), cardiopulmonary (intermediate), gastrointestinal and gustatory (rostral), and all states influenced by the region: emotional, behavioral, and autonomic (Craig, 2003).

Electron microscopic immunohistochemistry revealed dense labeling of polySia in the space around neuronal cell bodies, fibers, and dendrites (Fig. 2A). polySia-ir does not colocalize with GFAP-labeled astrocytes in the NTS (Bouzioukh et al., 2001a). The ultrastructural location is therefore consistent with polySia present in or on the fine processes of astrocytes that lack GFAP and/or within the extracellular space or adjacent to plasma membranes (Fig. 2Ai, Aii). The labeling pattern is consistent with the punctate rings of immunoreactivity that surround some neuronal cell bodies identified by light microscopy (Fig. 1Bi, Bii). polySia-ir was found on the rough endoplasmic reticulum and Golgi apparatus of some neuronal cell bodies (Fig. 2A) where it may be produced before being transported extracellularly to the cell surface (as described in the hypothalamus) (Theodosis et al., 1999). polySia-ir was also present adjacent to some synapses and in a subset of dendrites and axon terminals (Fig. 2B–E), consistent with the close association of polySia and synaptophysin revealed using immunofluorescence (Fig. 1Ci, Ci).

Enzymatic digestion of polySia enhances ionic currents of neurons in the NTS
To determine whether removal of sialic acids would alter the ionic conductance of NTS neurons, we first determined the time...
Figure 2. Ultrastructural location of polySia immunoreactivity. A, Ultrastructural analysis shows polySia-ir (white arrows) surrounding neuronal soma (Ne), proximal dendrites (de), and fine neuronal processes consistent with expression in fine processes of astrocytes (As) (white arrows). polySia-ir is also found in the rough endoplasmic reticulum and Golgi apparatus of some neurons (black arrows). Aii, polySia-ir occurs along the exterior surface of the plasma membrane of some astrocytic (As) and neuronal (Ne) soma (white arrows) indicating its presence in the extracellular space. Aiii, Dendrites (de) and axons are sheathed by polySia labeling. B, polySia-ir (white arrows) was found adjacent to some synapses (sy) likely in astrocytic processes, as a tripartite synapse. C–E, polySia-ir is found within some dendrites (de) and axon terminals (te) of neurons. Black arrows indicate postsynaptic densities.
no effect in endoNF-treated slices ($F_{(1/6)} = 0.30, p = 0.60, n = 4$; Fig. 4B, Bi), suggesting that BK channel function was perturbed by polySia removal. Current injections did not alter the number of action potentials generated ($F_{(1/35)} = 0.21, p = 0.65$; Fig. 5A, Ai) or the amplitude of the AHP ($t_{(18)} = 0.133, p = 0.90$; Fig. 5B) in keeping with a reduced ability of Ibtx to close BK channels following polySia digestion (Yang et al., 2015). These data indicate that IKDR and BK channel function are both altered by the removal of polySia.

The effect of endoNF on steady-state currents was voltage-dependent and significantly enhanced at the equilibrium potential for Ca$^{2+}$ (9 mV under the recording conditions), so we examined whether polySia altered Ca$^{2+}$ currents. We first omitted Ca$^{2+}$ from the external solution preventing Ca$^{2+}$ influx during depolarization. Under these conditions, enzymatic digestion of polySia still enhanced steady-state currents ($F_{(1/28)} = 5.40, p = 0.027, n = 15$; Fig. 4C) that were indistinguishable from those evoked by endoNF under normal levels of external Ca$^{2+}$ ($F_{(1/49)} = 0.002, p = 0.96$). Next, we substituted EGTA within the recording pipette for the fast Ca$^{2+}$ chelator BAPTA (10 mM) to assess the contribution made by internal Ca$^{2+}$ stores. We found that BAPTA eliminated the enhanced steady-state outward currents normally observed following polySia digestion ($F_{(1/28)} = 0.15, p = 0.70, n = 13$; Fig. 4D).

Together, these data indicate that removal of polySia from NTS neurons facilitated currents generated through K$^+$ channels, reduced inactivation of BK channels, and increased Ca$^{2+}$ via release from intracellular stores.

**Figure 3.** polySia removal enhances current voltage relationship within NTS neurons. **A**, Representative Western blot using Mab735 to detect polySia during slice recovery, and following incubation in aCSF (gray/black box), Neu (0.1 U/ml, blue box), or endoNF (1 μg/ml, green box). GAPDH was used as a loading control. **B**, Comparison of the amount of protein detected using Mab735 during slice recovery (black and gray), and incubation in aCSF, Neu, or endoNF. polySia-ir was significantly reduced after 60 min incubation in Neu (blue box, $p = 0.0039, n = 3$) or 45 min incubation in endoNF (green box, $p = 0.0042, n = 3$). Data are normalized to GAPDH with the zero time point value set to 1. Enzymatic removal of polySia in NTS brain slices increases steady-state outward currents. **C**, Typical current traces (depolarizing voltage step: Δ10 mV) from NTS neurons incubated in aCSF (black), or Neu (0.01 U/ml, light blue, 0.1 U/ml, dark blue, and 1 U/ml, aqua, respectively). **Ci**, Grouped data reveal that Neu (0.1 and 1 U/ml) significantly increased current density of neurons at depolarized potentials $≥ 0$ mV ($p = 0.0002, n = 16$ and $n = 11$, respectively). **D**, Grouped data show that incubation in endoNF at 1 μg/ml (dark green), but not 0.1 μg/ml (light green), significantly increased current density of neurons at depolarized potentials $≥ 0$ mV ($p < 0.0001, n = 36$). Data are mean ± SEM. **E**, Recovered cell (green) within the NTS surrounded by polySia-ir puncta (white) coating the soma and proximal dendrite. The recovered cell was recorded while superfused with aCSF (control). Schematic coronal section showing distribution of recorded neurons in the NTS recovered following incubation in aCSF or endoNF. **p < 0.01; ****p < 0.0001; ns, p > 0.05.
polySia contributes to glutamatergic viscerosensory afferent transmission in NTS

We next investigated whether polySia removal affected viscerosensory afferent transmission. In second-order NTS neurons, identified as receiving monosynaptic input from viscerosensory afferents, enzymatic cleavage of polySia reduced the fidelity of action potentials generated by afferent activation (Fig. 6A). Consistent with previous observations (Bailey et al., 2007), consecutive, 1 Hz minimal amplitude electric stimulation generated action potentials with 86.0 ± 5.1% fidelity under control conditions previous shown to be mediated by AMPA receptors. In the same neurons, following polySia digestion with endoNF, fidelity decreased to 31.0 ± 3.3% (t(10) = 9.297, p = 0.0007, n = 5; Fig. 6Bi). Furthermore, polySia digestion increased the latency of action potential propagation following stimulation of viscerosensory afferents (Fig. 6B) by 41.7% (8.79 ± 1.56 ms vs 5.12 ± 0.61 ms, t(4) = 3.96, p = 0.021, n = 5; Fig. 6Bi). No significant differences were observed in basal action potential parameters, includ-
ing membrane potential ($-51.60 \pm 2.14 \text{ mV}$ vs $-49.20 \pm 1.72 \text{ mV}$, $t_{(4)} = 2.59, p = 0.061, n = 5$), action potential amplitude ($92.03 \pm 7.27 \text{ mV}$ vs $93.18 \pm 3.50 \text{ mV}$, $t_{(4)} = 0.27, p = 0.80, n = 5$), half-width ($1.54 \pm 0.30 \text{ ms}$ vs $1.10 \pm 0.16 \text{ ms}$, $t_{(4)} = 2.40, p = 0.074, n = 5$), rise time ($90\%$–$10\%$; $1.72 \pm 0.50 \text{ ms}$ vs $1.68 \pm 0.38 \text{ ms}$, $t_{(4)} = 0.13, p = 0.91, n = 5$), spike threshold ($-39.80 \pm 2.22 \text{ mV}$ vs $-33.31 \pm 0.87 \text{ mV}$, $t_{(4)} = 2.58, p = 0.061, n = 5$), or decay time ($T_{90\%-10\%}$; $1.96 \pm 0.21 \text{ ms}$ vs $1.64 \pm 0.17 \text{ ms}$, $t_{(4)} = 1.40, p = 0.23, n = 5$). These data suggest that polySia expression enables monosynaptic transmission from viscerosensory afferents onto NTS neurons.

As the fidelity of action potential transmission is perturbed following polySia removal in the NTS, we investigated whether excitatory drive from viscerosensory afferents would similarly decline. As described previously (Doyle and Andresen, 2001), suprathreshold electrical stimuli applied to viscerosensory afferents evoke large monosynaptic EPSCs (eEPSCs), identified by low failure rates and jitters ($200 \mu s$; $140.60 \pm 13.63 \mu s$, $n = 12$). Consistent with effects on action potential probability and latency, enzymatic digestion of polySia increased eEPSC latency by $31.1\%$ ($4.97 \pm 0.44 \text{ ms}$ vs $3.79 \pm 0.38 \text{ ms}$, $t_{(9)} = 3.51, p = 0.0066, n = 10$; Fig. 7A, A1), and decreased the amplitude of eEPSCs recorded in voltage-clamp mode by $54.4\%$ ($198.60 \pm 31.14 \text{ pA}$ vs $412.20 \pm 59.93 \text{ pA}$, $t_{(9)} = 5.527, p = 0.0004, n = 10$; Fig. 7A, Aii). In contrast, jitter ($159.90 \pm 7.30 \mu s$ vs $134.60 \pm 10.06 \mu s$, $t_{(9)} = 2.242, p = 0.0517, n = 10$), paired-pulse ratio (EPSC2/EPSC1;
Figure 7. polySia removal in the NTS reduces glutamatergic synaptic input. **A**, Typical current traces of monosynaptically connected NTS neurons with eEPSCs generated following visceral sensory afferent stimulation (5 Hz) before (black) and after (green) endoNF incubation. Individual trials are represented in gray and light green, respectively. **Ai**, **Aii**, Grouped data show that enzymatic digestion of polySia increased latency by 34.2% (\(p = 0.0098, n = 9\)) and decreased eEPSC amplitude by 54.4% (\(p = 0.0002, n = 9\)). **B**, Typical current traces of sEPSCs in monosynaptically connected NTS neurons before (black) and after (green) endoNF incubation. **Bi**, **Bii**, Grouped data show that removal of polySia decreased frequency by 47.2% (\(p = 0.0074, n = 5\)) and amplitude by 26% (\(p = 0.034, n = 5\)). **C**, Current trace of NMDA-mediated sustained currents in monosynaptically connected NTS neurons before (black) and after (green) endoNF incubation. **Ci**, Sustained current 100 ms (hashed vertical line) after initial stimulus. **Cii**, Grouped data show that enzymatic digestion of polySia increased sustained current by 42% (\(p = 0.0031, n = 4\)) in response to high-frequency stimulation of the solitary tract. Stimulus artifacts and current response to first shock were truncated to aid visual representation. **D**, Current traces showing mEPSCs recorded before (black) and after endoNF treatment (green) with both TTX and GBZ present in the bath. CNQX (gray) was added to the superfusate at the end of experimentation. **Di**, Frequency distribution over time shows a progressive decrease in mEPSC frequency (10 s bins) following addition of endoNF to the perfusate. **Dii**, **Diii**, Grouped data show that enzymatic digestion of polySia decreased frequency by 71% (\(p = 0.04, n = 4\)) but had no effect on mEPSC amplitude (\(p = 0.156, n = 4\)). Data are mean ± SEM. *\(p < 0.05\); **\(p < 0.01\); ***\(p < 0.001\); ns, \(p > 0.05\).
0.53 ± 0.08 vs 0.58 ± 0.05, \( t_{(5)} = 1.54, p = 0.18, n = 6 \), and eEPSC decay tau (\( T_{90\% \text{--} 10\%} \)) 5.67 ± 1.70 ms vs 5.65 ± 0.60 ms, \( t_{(5)} = 0.30, p = 0.78, n = 6 \) remained unaffected following polySia digestion.

In the same neurons, we examined whether polySia was required for the maintenance of eEPSCs, arising from spontaneous glutamatergic release. Enzymatic digestion of polySia significantly decreased both the frequency of eEPSC by 58.80% (3.80 ± 2.30 Hz vs 7.70 ± 2.80 Hz, \( t_{(4)} = 5.01, p = 0.0074, n = 5 \), and amplitude by 27.80% (22.34 ± 5.10 pA vs 30.21 ± 3.65 pA, \( t_{(4)} = 3.18, p = 0.034, n = 5 \); Fig. 7B, Bi). Together, these data indicate the importance of polySia expression within the NTS in facilitating excitatory synaptic transmission from visceral sensory afferents mediated by AMPA receptor activation.

In a subset of monosynaptically connected neurons, we investigated the effect of polySia removal on NMDA-mediated sustained currents, recently identified in second-order NTS neurons (Zhao et al., 2015). Neurons were voltage clamped at −60 mV and recorded in Mg\(^{2+}\)-free aCSF to remove the Mg\(^{2+}\) block on NMDA receptors (Mayer et al., 1984). In 4 of 5 neurons tested, stimulating the solitary tract at 50 Hz consistently evoked large sustained currents (Fig. 7C,G,Cl), that were not observed at lower stimulating frequencies (1 Hz, data not shown). Enzymatic digestion of polySia increased the current generated to high-frequency stimulation of the solitary tract by 42 ± 4.80% \( (t_{(3)} = 8.758, p = 0.0031, n = 4 \); Fig. 7Ci).

Collectively, these data suggest that the fidelity of excitatory synaptic transmission during high- and low-frequencyafferent input is maintained by polySia expression, and that its removal may alter the appropriate charge required to open and close AMPA and NMDA receptors as previously suggested (Vaithianathan et al., 2004; Hammond et al., 2006).

### polySia contributes to presynaptic glutamate release in the NTS

To determine whether polySia removal impacts neurotransmission post-synaptically or presynaptically as recorded, in the presence of TTX (10 \( \mu M \)) and GBZ (25 \( \mu M \)), eEPSCs, and measured responses before and during polySia digestion (Fig. 7D, Di). Enzymatic digestion of polySia decreased eEPSC frequency by 68.75% (endoNF, 4.00 ± 0.57 Hz vs control, 13.75 ± 3.30, \( t_{(3)} = 3.47, p = 0.040, n = 4 \); Fig. 7Div) without altering amplitude (62.00 ± 15.70 pA vs 74.50 ± 22.08 pA, \( t_{(3)} = 1.88, p = 0.156, n = 4 \); Fig. 7Dii). CNQX was added at the end of experimentation demonstrating that eEPSCs were dependent on non–NMDA-type glutamate receptors (Fig. 7D). These data indicate that removal of polySia reduces glutamatergic release from presynaptic terminals at NTS neurons, and this mechanism likely contributes to the reduction in visceral sensory afferent transmission (both evoked and spontaneous) seen following polySia digestion.

### Enzymatic cleavage of polySia within the NTS increases sympathetic nerve discharge in vivo

Finally, as multiple pathways emerge from the NTS that influence presynaptic neurons regulating the sympathetic outflow (Guyenet, 2006), we determined whether the changes to intrinsic neuronal properties and/or visceral sensory afferent transmission resulting from polySia digestion within the NTS in vitro, would be sufficient to alter function in vivo. sSNA and arterial pressure recorded before and after microinjection of neuraminidases directly into the caudal and intermediate NTS, in urethane-anesthetized rats. Bilateral microinjections of endoNF (2 \( \mu g/\mu l \), total volume 800 nl) elicited sympathoexcitation (Fig. 8A; \( F_{(1,8)} = 34.06, p = 0.0004 \)) with peak increases in sSNA of 140.80 ± 7.63% compared with control (saline, 96.50 ± 4.48%, \( t_{(104)} = 4.991, p < 0.0001, n = 6 \); Fig. 8Ai). Brains were removed at the end of each experiment and the degree of polySia digestion determined using Western blot analysis. polySia was present following PBS microinjection but absent following endoNF microinjection (0.94 ± 0.05 a.u. vs 0.09 ± 0.02 a.u., \( t_{(21)} = 5.71, p < 0.0001, n = 6 \); Fig. 8Bi, Biv). Microinjection of the neuraminidase targeting only terminal \( \alpha 2-3 \) and \( \alpha 2-6 \) sialic acids (\( \alpha 2-3 + \alpha 2-6 \) Neu, 0.1 U/\( \mu l \)) had no effect on sSNA (\( F_{(1,8)} = 2.580, p = 0.15, n = 5 \); Fig. 8Aii, Aiv) and similarly did not alter the expression of polySia relative to control (\( t_{(21)} = 0.81, p > 0.99, n = 5 \); Fig. 8Bi, Biv).

polySia also terminates \( \alpha 2-3 \) and \( \alpha 2-6 \) sialic acid–linked glycans (Schnaar et al., 2014), which would be untouched by \( \alpha 2-3 + \alpha 2-6 \) Neu; we therefore determined the combined effect of enzymatically removing polySia together with \( \alpha 2-3 \) and \( \alpha 2-6 \) sialic acid residues by combining both enzymes (\( \alpha 2-3 + \alpha 2-6 \) Neu + endoNF). Microinjection of the combined enzymes bilaterally caused rapid sympathoexcitation (Fig. 8Aii; \( F_{(1,7)} = 20.43, p = 0.0027 \) with a peak effect of 162.50 ± 13.73% \( (t_{(11)} = 4.760, p < 0.0001, n = 5 \); Fig. 8Aii) and in support of a specific role for polySia, no polySia was detected at the protein level (0.01 ± 0.02 a.u. vs 0.94 ± 0.05 a.u., \( t_{(21)} = 5.90, p < 0.0001, n = 5 \); Fig. 8Bii, Biv). Similarly, microinjection of Neu (0.1 U/\( \mu l \)) also produced rapid sympathoexcitation \( (F_{(1,8)} = 68.18, p < 0.0001) \) and peaked at a higher level (179.33 ± 14.56%, \( t_{(10)} = 4.315, p = 0.0005, n = 6 \); Fig. 9A, Ai) that was significantly greater than endoNF alone \( (p = 0.041, Mann–Whitney U test, n = 6 \); Fig. 9B) but did not differ from that evoked by \( \alpha 2-3 + \alpha 2-6 \) Neu + endoNF \( (p = 0.628, Mann–Whitney U test, n = 5 \).

The effects on arterial pressure were more variable following enzymatic digestion of polySia. Neu evoked the largest and most rapid sympathoexcitation (see above) which acutely elevated mean arterial pressure (MAP) compared with control \( (15 \text{ min}: 152 \pm 10.97 \text{ vs } 87.75 \pm 7.32 \text{ mmHg, } t_{(13)} = 4.425, p = 0.0014, n = 6 \); Fig. 9A, B, Ci). This MAP increase however, returned to baseline within 60 min \( (108 \pm 3.1 \text{ vs } 92.5 \pm 7.5 \text{ mmHg, } t_{(13)} = 1.91, p = 0.157, n = 6 \); Fig. 9Ci). As endoNF evoked smaller and slower sympathoexcitation, little to no effect on MAP was seen acutely \( (15 \text{ min}: 98.24 \pm 9.11 \text{ vs } 87.75 \pm 7.32 \text{ mmHg, } t_{(13)} = 0.713, p = 0.97, n = 6 \); Fig. 9Ci) or at 60 min \( (95.02 \pm 6.38 \text{ vs } 92.5 \pm 7.5 \text{ mmHg, } t_{(13)} = 0.301, p > 0.99, n = 6 \); Fig. 9Ci).

Together these results demonstrate that removal of polySia in the NTS increases sympathetic nerve activity, which is consistent with a net disfacilitation in the NTS or experimental visceral deafferentation (Iggo and Vogt, 1962; Fagius et al., 1985). Thus, our findings indicate that polySia expression within the NTS is required to maintain appropriate (i.e., ”normal”) levels of sympathetic outflow. Figure 10 depicts the cellular mechanisms and network effects modified by polySia in the NTS highlighting the consequences of polySia’s removal.

### Discussion

We demonstrate that polySia expression in the dorsal medulla is required for normal excitatory neurotransmission within the NTS, and that its disruption is sufficient to acutely increase sympathetic outflow. We draw these conclusions based on the following observations: First, neurons in the NTS were eneathed by polySia, expressed predominantly in or on the fine processes of astrocytes and within the extracellular space. Second, enzymatic removal of polySia–enhanced currents in NTS neurons, mediated by changes at IKDR and BK channels, and altered release of Ca\(^{2+}\) from internal stores. Third, enzymatic removal of
polySia reduced AMPA-mediated excitatory transmission following viscerosensory afferent stimulation by decreasing evoked EPSCs, attenuating action potential generation and propagation and diminishing spontaneous and mEPSCs, whereas high-frequency stimulus-evoked NMDA-dependent sustained currents were increased. Finally, enzymatic removal of polySia from the NTS in vivo increased splanchnic sympathetic nerve activity, whereas microinjection of sialidases that do not target polySia had little effect. Collectively, our studies indicate that polySia plays a hitherto unrecognized role in the modulation of neural transmission within the NTS, and demonstrate that polySia expression is required for the appropriate processing of viscerosensory afferent activity and its transmission to downstream networks, including the sympathetic outflow.

We extend earlier studies that describe the location of polySia in the CNS (Bonfanti et al., 1992; Bouzioukh et al., 2001a, b) demonstrating abundant polySia expression throughout the rostrocaudal extent of the NTS. Our ultrastructural analysis revealed polySia expression at sites that influence neurotransmission: in the extracellular space and intricate processes of astrocytes that

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**Figure 8.** polySia removal in the NTS increases sympathetic nerve activity. **A.** Integrated and smoothed representative traces of sSNA recorded in urethane-anesthetized, paralyzed, and ventilated rats following saline (black), endoNF (green 2 μg/μl), α2–3 + α2–6 Neu (magenta, 0.1 U/μl), or α2–3 + α2–6 Neu + endoNF (orange) microinjections bilaterally into the NTS. Gray represents the period of injection. **Ai.** Grouped data show that endoNF (n = 6) and α2–3 + α2–6 Neu + endoNF (n = 5) significantly increased sSNA by 40.8% and 62.5%, respectively (p < 0.0001). Microinjection of α2–3 + α2–6 Neu alone had no effect compared with control (p = 0.15, n = 5). **B.** Western blots of NTS dissected from the animals in (A) following microinjection of control, endoNF (Bi, 2 μg/μl, green), α2–3 + α2–6 Neu (Bii, 0.1 U/μl, magenta), or α2–3 + α2–6 Neu + endoNF (Biii, orange). **Biv.** Grouped data showing quantitative changes in polySia protein (detected using Mab735) from animals in B. polySia-ir was virtually absent following microinjection of either endoNF (green box, p < 0.0001, n = 6) or α2–3 + α2–6 Neu + endoNF (orange box, p < 0.0001, n = 5), and was not altered following microinjection of α2–3 + α2–6 Neu alone (magenta box, p > 0.99, n = 5). Data are mean ± SEM. **p < 0.01; ***p < 0.001; ****p < 0.0001; ns, p > 0.05.
envelop neurons and synapses, as well as in some dendrites and axon terminals, consistent with our synaptophysin/polySia double labeling (Fig. 10A). Supporting our finding of polySia expression in astrocytic fine processes, blockade of microtubular function in the neurosecretory hypothalamus caused accumulation of polySia in astrocyte cell bodies (Theodosis et al., 1999, 2008). Expression around neurons found here is consistent with previous studies in the hippocampus, striatum, and cortex where polySia is linked with synaptic plasticity (Muller et al., 1996; Uryu et al., 1999; Eckhardt et al., 2000; Hildebrandt and Dityatev, 2015). Enzymatic digestion of polySia has no effect on presynaptic or postsynaptic neuronal structures examined by electron microscopy (Theodosis et al., 1999; Brusés et al., 2002), suggesting that functional effects result from disruption of signaling mechanisms, rather than major morphological changes.

In exploring the cellular mechanisms influenced by polySia in the NTS, we found that enzymatic removal of polySia increased TEA-sensitive currents, inhibited the closure of BK channels, and produced effects consistent with the increased release of Ca^{2+} from intracellular stores (Fig. 10A). The effects of TEA differed to those of Ibtx, indicating effects at multiple K^+ channels, including at delayed rectified K^+ channels, common in NTS neurons (Andresen and Kunze, 1994). Functional effects of NTS desialylation were not unexpected given that voltage-gated K^+ channels present on NTS neurons are heavily glycosylated, often with glycans likely capped by sialic acids (Cartwright and Schwalbe, 2009; Ednie and Bennett, 2012). A range of effects of desialylation have been described in other cell types, including both depolarizing and hyperpolarizing shifts in channel activation (Ednie and Bennett, 2012; Scott and Panin, 2014), which appear dependent upon the cell type examined and the channel isoform investigated. These complications arise as desialylation of the same ion channel in different cell types can produce varying effects (Ednie and Bennett, 2012; Scott and Panin, 2014) and channel isoforms can also vary in the glycan structures they carry (Schwalbe et al., 2008). Curiously astrocytes express abundant voltage-gated K^+ channels (Contet et al., 2016), so it is possible that polysialylation may also impact K^+ spatial buffering. Although both sialic acids and polySia are associated with voltage-gated Na^+ channels in rat brain (Zuber et al., 1992; Bennett et al., 1997; Ednie and Bennett, 2012) we saw no impact of polySia removal on action potential properties generated by current injection or following afferent stimulation.

We found that removal of polySia disrupted information transfer from visceral sensory afferents as evoked and spontaneous AMPA-dependent EPSCs were reduced, and NMDA sustained currents increased, indicating a role for polySia in regulating excitatory neurotransmission (Fig. 10A). As visceral sensory afferents innervate both somata and dendrites of NTS neurons (Anders et al., 1993), the astrocytic, neuronal, and extrasynaptic location of polySia seen here, together with the close association with synaptophysin shown by us and others (Bouzioukh et al., 2001a), are consistent with such a role. It is plausible that polySia on astrocytes may also influence synaptic or extrasynaptic glutamate availability as astrocytes express abundant glutamate transporters such as EAAT2 responsible for 90% of glutamate reuptake in brain.
Figure 10. Overview depicting the structural location of polySia as well as the cellular mechanisms modified and functional consequences following removal of polySia from the NTS. A, Astrocytic, terminal, and extrasynaptic expression of polySia influences Kv, intracellular calcium, and viscerosensory afferent glutamatergic transmission: during low-frequency input (magenta stars), and high input (pink stars), maintaining appropriate transmission through the NTS. B, Schematically, the local and network impact of polySia in the NTS under in vivo (Figure legend continues.)
Within the NTS, variations in synaptic strength usually result from differences in the number of contact or release sites between afferent axons and postsynaptic membranes, rather than glutamate release probability or uptake capacity (Bailey et al., 2006b; Peters et al., 2008). Reduced action potential generation has been demonstrated in the NTS when K⁺ channels are activated before visceral sensory afferent stimulation (Bailey et al., 2006a, 2007), or when glutamate release sites from single afferents are reduced (Bailey et al., 2006b). It appears paradoxical that polysia is not present within synapses in the NTS, given that its removal perturbs all forms of afferent excitation (e/s/mEPSC). However, polysia likely modulates transmission via effects directly at K⁺ channels, and/or by altering availability of glutamate to the postsynaptic density, or via mechanisms at multiple release site recently described in NTS neurons (Fawley et al., 2016).

Also in keeping with effects seen here on evoked transient and sustained currents, polysia can modulate AMPA and NMDA receptor function (Vaiithianathan et al., 2004; Hammond et al., 2006; Varbanov and Dityatev, 2017). Both receptor types are present within the NTS and are activated by differing afferent input (Aylwin et al., 1997; Zhang and Mifflin, 1998), with low-frequency stimulation evoking a large transient AMPA-mediated current (Andresen and Yang, 1990), and high-frequency stimulation (>5 Hz) a sustained NMDA-mediated current (Zhao et al., 2015). In support of our findings, activation of AMPA receptors in reconstituted lipid bilayers, in the presence of bacterially derived polysia, potentiated currents (~67%) and increased the opening probability of these receptors (~360%) (Vaiithianathan et al., 2004), with opposing effects observed at NMDA receptors (decreased opening probability in the presence of polysia) (Hammond et al., 2006). Our findings therefore support previous suggestions that the anionic charge of polysia directly influences the positive amino acid residues of AMPA and NMDA receptors, as suggested for other polyanionic polysaccharides, such as heparin (Hall et al., 1996; Sinnarajah et al., 1999) and dextran (Suppiramaniam et al., 2006; Chicoine and Bahr, 2007). Whether GluN2B subunits containing NMDA receptors are targeted by removal of polysia as found previously (Kochlamazashvili et al., 2010) remains to be determined. Nevertheless, it is plausible that the anionic polysia provides optimal conditions for regulating glutamatergic transmission in the NTS.

The effects seen following polysia removal at the single neuron strongly support our findings in the whole animal, where enzymatic removal of polysia from the NTS region increased splanchnic sympathetic nerve activity (Fig. 10B). This finding is consistent with net disfacilitation of the NTS, effectively reducing information throughput resulting in sympathoexcitatory transmission via increased activity of premotor neurons (Pilowsky and Goodchild, 2002). Baroreceptor afferents provide tonic activation of the NTS altering activity on a heartbeat to heartbeat basis. Under basal conditions (as recorded here), baroreceptor firing frequency is low, favoring AMPA receptor activation with minimal input from NMDA receptors (Seagard et al., 1990; Gordon and Leone, 1991). When baroreceptor afferent traffic is reduced, sympathetic nerve activity increases (Sved et al., 1997) suggesting that polysia could influence such transmission. The inverse relationship between vagal afferent traffic and polysia expression in the NTS previously described (Bouzioukh et al., 2001a, b), and the sympathoexcitatory effect evoked by polysia removal seen here, appears to provide a mechanism to explain why stimulation of vagal afferents increases lumbar sympathetic nerve activity (Sun and Guyenet, 1987). The lack of a consistent blood pressure response following removal of polysia may be due to a threshold effect where blood pressure is not increased by the small, slow changes in sympathetic nerve activity induced by endo N alone, whereas Neu induced larger and more rapid changes in sympathetic nerve activity elevating blood pressure. Alternatively, polysia removal in the NTS may result in opposing changes in different vascular beds or influence splanchnic sympathetic fibers that innervate targets other than the vasculature. Nevertheless, the data indicate that sympathetically outflow is dependent on the expression of polysia within the NTS, which can be modified by changes to vesicerosensory afferent traffic.

Targeting polysia consistently evoked sympathoexcitation. However, effects were enhanced by exo-neuraminidases that target α2–3, α2–6, in addition to α2–8 linked sialic acids, suggesting a role for other types of sialylation in regulating sympathetically outflow. However, endoNP only cleaves polysia polymers of five to eight α2–8 linked sialic acids (Stummeyer et al., 2005), potentially leaving some sialic acids (including single, di, or oligo sialic acids) that could be cleaved by the other enzymes used. Our Western blotting would not have detected these residual sialic acids because Mab735 recognizes octamers of α2–8 linked sialic acids (Evans et al., 1995; Nagae et al., 2013).

Collectively, our data show that removal of polysia alters neuronal properties and transmission of vesicerosensory excitatory traffic through the NTS and that these effects alter transmission to downstream networks increasing sympathetic outflow (Fig. 10). This study expands the repertoire of signaling molecules, particularly those located on the cell surface or in astrocytes, which influence information transfer within sensory nuclei. The NTS governs autonomic, hormonal, and behavioral activity indicative of the wide spectra of functions that polysia may impact (Craig, 2003). Furthermore, the findings described here may define the neuronal mechanisms modified by polysia in higher brain regions (Rutishauser and Landmesser, 1996; Rutishauser, 2008) as well as at several sensory nuclei expressing abundant polysia (Bonfanti et al., 1992; El Maarouf et al., 2005). Finally, we demonstrate the importance of glycosylation modifications, such as sialylation, in altering neuronal function and show the importance of the level of polysia expression for appropriate information processing in the dorsal medulla, which ultimately regulates the activity of multiple downstream neural networks.

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Bonfanti et al., 1992; El Maarouf et al., 2005). Finally, we demonstrate the importance of glycosylation modifications, such as sialylation, in altering neuronal function and show the importance of the level of polysia expression for appropriate information processing in the dorsal medulla, which ultimately regulates the activity of multiple downstream neural networks.

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