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GLIA DETERMINE THE COURSE OF BDNF-MEDIATED DENDRITOGENESIS AND PROVIDE A SOLUBLE INHIBITORY CUE TO DENDRITIC GROWTH IN THE BRAINSTEM

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Abstract

Cardiorespiratory control neurons in the brainstem *nucleus tractus solitarius* (NTS) undergo dramatic expansion of dendritic arbors during the early postnatal period, when functional remodeling takes place within the NTS circuitry. However, the underlying molecular mechanisms of morphological maturation of NTS neurons are largely unknown. Our previous studies point to the neurotrophin brain-derived neurotrophic factor (BDNF), which is abundantly expressed by NTS-projecting primary sensory neurons, as a candidate mediator of NTS dendritogenesis. In the current study, we used neonatal rat NTS neurons *in vitro* to examine the role of BDNF in the dendritic development of neurochemically-identified subpopulations of NTS neurons. In the presence of abundant glia, BDNF promoted NTS dendritic outgrowth and complexity, with the magnitude of the BDNF effect dependent on neuronal phenotype. Surprisingly, BDNF switched from promoting to inhibiting NTS dendritogenesis upon glia depletion. Moreover, glia depletion alone led to a significant increase in NTS dendritic outgrowth. Consistent with this result, astrocyte-conditioned medium (ACM), which promoted hippocampal dendritogenesis, inhibited dendritic growth of NTS neurons. The latter effect was abolished by heat-inactivation of ACM, pointing to a diffusible astrocyte-derived negative regulator of NTS dendritic growth. Together, these data demonstrate a role for BDNF in the postnatal development of NTS neurons, and reveal novel effects of glia on this process. Moreover, previously documented dramatic increases in NTS glial proliferation in victims of sudden infant death syndrome (SIDS) underscore the importance of our findings and the need to better understand the role of glia and their interactions with BDNF during NTS circuit maturation. Furthermore, while it has previously been demonstrated that the specific effects of BDNF on dendritic growth are context-dependent, the role of glia in this process is unknown. Thus, our data carry important implications for mechanisms of dendritogenesis likely beyond the NTS.

Keywords

Astrocyte; GAD67; GFAP; Nucleus tractus solitarius; TH; VGlut2

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Neurons in the brainstem *nucleus tractus solitarius* (NTS) carry vital reflexes and are critical to cardiorespiratory homeostasis (Andresen and Kunze, 1994; Guyenet, 2006). During the early postnatal period, these neurons experience remarkable expansion of dendritic arbors (Kalia et al., 1993; Vincent and Tell, 1999). Yet, the mediators and mechanisms of dendritogenesis in the NTS remain largely unidentified.

Based on studies in other parts of the nervous system, it is thought that the ultimate features of dendritic arbors are guided by a complex interplay among cell intrinsic programs and external cues (Kim and Chiba, 2004; Parrish et al., 2007; Cline and Haas, 2008). One well-established external cue is the neurotrophin brain-derived neurotrophic factor (BDNF; McAllister, 2000; Dijkhuizen and Ghosh, 2005). However, the effects that BDNF elicits on dendritic morphology are far from straightforward. For example, in pyramidal neurons of the visual cortex, the length and complexity of basal dendrites are increased by BDNF in layer 4 (McAllister et al., 1995; McAllister et al., 1997), but decreased in layer 6 (McAllister et al., 1997). The opposing effects of BDNF on dendritic growth can also depend on the sub-cellular localization of receptor activation (Lom and Cohen-Cory, 1999; Lom et al., 2002), the type of signaling activated downstream of BDNF (Ohira and Hayashi, 2009), as well as other features, such as developmental time point (Gorski et al., 2003).

Recent studies indicate that dendritic morphogenesis can also be regulated by astrocytes (Procko and Shaham, 2010). Whether any interactions exist between BDNF and astrocytes to influence the overall dendritic arbor is currently unknown. In hippocampal cultures, an unidentified diffusible factor released by astrocytes promotes inhibitory synapse formation in a mechanism dependent on neuronal BDNF release and signaling through TrkB, the high-affinity receptor for BDNF (Elmariah et al., 2005). The latter suggests a complex interplay between neurotrophins and glia that likely represents a nervous system-wide phenomenon. This begs the question: are some BDNF effects on dendritic morphology influenced by glia?

Our previous studies show that primary visceral sensory neurons, which form direct synaptic connections with second-order sensory neurons in the NTS, express and release BDNF (Martin et al., 2009). In fact, BDNF is abundantly expressed in visceral afferents and their central terminals in the NTS (Martin et al., 2009; Kline et al., 2010), while the TrkB receptor is found in NTS neurons (Balkowiec et al., 2000; Kline et al., 2010; Clark et al., 2011). Thus, BDNF is strategically positioned to mediate dendritic morphogenesis in NTS visceral sensory pathways.

In the present study, we have tested the hypothesis that BDNF regulates dendritic outgrowth and complexity of NTS neurons, using dissociate cultures of neurochemically-identified subpopulations. We show that, in mixed neuron-glia cultures, BDNF stimulates NTS dendritogenesis in a neuronal phenotype-dependent manner. Following glia depletion, BDNF switches from promoting to inhibiting NTS dendritogenesis. Moreover, astrocyte-conditioned medium exerts a potent inhibitory effect on the dendritic growth of NTS neurons, while promoting dendritogenesis of hippocampal neurons. These data have important implications for the possible pathogenesis of sudden infant death syndrome (SIDS).

EXPERIMENTAL PROCEDURES

Animals

Sprague-Dawley rats (Charles River Laboratories) were used in these studies. All procedures were approved by the Institutional Animal Care and Use Committee of the Oregon Health and Science University.

Primary dissociate cultures of the NTS region

Postnatal day (P) 2–3 Sprague-Dawley rat pups were euthanized with an intraperitoneal injection of Euthasol (0.1 mg/kg) and decapitated. Whole medullas were rapidly and aseptically dissected from each animal into ice-cold $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Dulbecco's phosphate-buffered salt solution (PBS; Mediatech). Subsequently, a block dissection of the *nucleus tractus solitarius* (NTS) was performed as previously described (Fitzgerald et al., 1992). Briefly, the NTS block was obtained by making one cut at the commissural NTS and the other at the level of obex. Next, area postrema, and cuneate and gracile nuclei were all cut out of the block with a fine scalpel blade. Every effort was made to limit the block to the NTS, but due to the nature of the block dissection, some adjacent areas may have been included. Tissue blocks were minced using a scalpel blade, and placed into 0.1–0.15% crystallized trypsin 3x (Worthington Biochemical Corporation) that was dissolved in pre-equilibrated $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free Hank's balanced salt solution (CMF-HBSS, Mediatech) for 30 min in a tissue culture incubator (37°C; humidified 95% air/5% CO_2). Next, tissue was rinsed in 0.1% soybean trypsin inhibitor (Worthington) dissolved in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -containing Dulbecco's PBS (Mediatech), followed by a rinse in plating medium, both at room temperature. Culture medium consisted of Neurobasal-A (NBA, Invitrogen), B-27 (Invitrogen), L-glutamine (2 mM, Invitrogen), and an antibiotic mixture of penicillin, streptomycin, and neomycin (PSN, Invitrogen). For plating, the medium was supplemented with fetal bovine serum (FBS; 10%, HyClone), which was subsequently removed from all cultures after 24 hrs. Tissue was placed in 2 mL of plating medium and dissociated through approximately four fire-polished Pasteur pipettes of decreasing tip diameter. Larger debris was allowed to settle for approximately 5 min and the supernatant was transferred to a new tube. The final cell density was adjusted to approximately 500,000 viable cells/mL based on hemacytometer cell counts in Trypan blue-stained samples. In order to reduce the number of glia at plating, cells were first pre-plated two times onto 15 mm diameter, untreated glass coverslips (Fisher Scientific) in 24-well tissue culture plates (Corning, 250,000 cells per well) for 30 min each, in the tissue culture incubator. After pre-incubation on untreated glass to allow binding of glia, the medium containing unattached cells was removed from the wells and plated into 24-well plates fitted with poly-D-lysine-coated (0.1 mg/mL, Sigma-Aldrich) glass coverslips (15 mm diameter, Fisher Scientific). After 24 hrs post-plating, all cell medium was replaced with serum-free neuronal maintenance medium. For glia-depleted conditions, cytosine β -D-arabinofuranoside (AraC; 5 μM , Sigma-Aldrich) was added to the serum-free maintenance medium to prevent glial cell proliferation.

BDNF treatment

BDNF (Promega) from a freshly thawed stock solution (100 $\mu\text{g}/\text{mL}$ in PBS, stored at -80°C) was diluted in the neuronal maintenance medium with or without AraC, to a final concentration of 200 ng/mL, as in a previous study of similar nature (Singh et al., 2008). Cultures were switched to either BDNF- or PBS (control)-containing medium at 4 days in vitro (DIV) and maintained for three days until fixed on the 7th DIV.

Primary dissociate hippocampal cultures

Hippocampi were dissected from embryonic day (E) 20 Sprague-Dawley rats as previously described (Banker and Cowan, 1977). Tissue was enzymatically treated and dissociated as described above for the *primary dissociate cultures of the NTS region*, except that 0.1% trypsin was applied for 15 min. The final cell density was adjusted to approximately 100,000 viable cells/mL. Cells were then plated into wells of a 24-well tissue culture plate (50,000 cells/well) containing glass coverslips (12 mm diameter, Fisher Scientific) that were pre-coated with poly-D-lysine (0.1 mg/mL).

Astrocyte cultures and preparation of ACM

Primary astrocyte cultures of either the NTS region or cortex were prepared from P2-3 Sprague-Dawley rats using a protocol adapted from a previously described procedure (McCarthy and de Vellis, 1980). Briefly, tissue was dissected and treated with enzyme as described above for the *primary dissociate cultures of the NTS region*. Following enzyme treatment, tissue was dissociated in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS and PSN that was pre-equilibrated in the tissue culture incubator. Cells were plated into 12.5 cm² tissue culture flasks (BD Biosciences) at a density of $1.0\text{--}1.2 \times 10^6$ viable cells per flask. Cultures were maintained in the tissue culture incubator, replacing the medium every 2–3 days until they reached confluency (10–16 days). Once cultures were confluent, flasks were sealed and shaken for 18–20 hrs on an orbital shaker (250 rpm at 37°C). The next day, flasks were rinsed and trypsinized (0.25% trypsin dissolved in 2 mL of pre-equilibrated CMF-HBSS) for 2 min to lift the astrocyte-enriched cell monolayer. Cells were rinsed two times in medium before their final re-suspension in 4–6 mL of medium and reseeded into 8–12 wells of a 24-well tissue culture plate. Re-seeded cultures typically reached confluency 3–5 days later and consisted of >95% astrocytes (the fraction of cells with DAPI-stained nuclei that shows glial fibrillary acidic protein (GFAP)-immunoreactivity). Prior to generating ACM, astrocyte cultures were switched to serum-free neuronal maintenance medium. Every 1–2 days, ACM was collected and pooled from all wells of the astrocyte cultures to be used immediately.

ACM treatment of cultures and heat-inactivation of ACM

NTS cultures were treated with NTS ACM, cortical ACM, or control medium. Control medium was the neuronal maintenance medium (please see the *Primary dissociate cultures of the NTS region* section above for details) that was pre-incubated in untreated 24-well tissue culture plates for the same amount of time that ACM was generated by astrocytes. All media were supplemented with AraC (5 μ M) to prevent glial cell proliferation. For ACM heat-inactivation studies, NTS ACM and control medium were incubated for 30 min in a 55°C water bath followed by equilibration in the tissue culture incubator prior to being supplemented with AraC and used on cultures. The cultures were treated with freshly generated ACM or control every 24 hrs between 4 DIV and 7 DIV, when the cultures were fixed.

For hippocampal cultures, the entire medium was replaced with NTS ACM, cortical ACM, or control medium 3 hrs after plating. The treatment was repeated approximately every 24 hrs until cultures were fixed at 5 DIV. This experiment was performed side-by-side with NTS cultures that were treated with NTS ACM from the same pool.

Immunocytochemistry

Cultures were fixed and immunostained according to our previously published procedures (Martin et al., 2009), except that all primary antibodies were incubated 18–24 hrs at 4°C. The following primary and secondary antibody pairs were used: i) Chicken anti-MAP2 (1:5,000; Millipore) with Goat anti-chicken Alexa 488 (1:500; Invitrogen); ii) Rabbit anti-TH (1:1,000; Pel-Freez; Chan and Sawchenko 1995) with Goat anti-rabbit Alexa 647 (1:500; Invitrogen); iii) Mouse anti-GAD67 (1:1,000; Millipore, clone 1G10.2; Fong et al., 2005) with either Goat anti-mouse Cy3 (1:500; Jackson ImmunoResearch) or Goat anti-mouse Alexa 647 (1:500; Invitrogen); iv) Guinea pig anti-VGlu2 (1:20,000; Millipore; Rosin et al., 2006) with Goat anti-guinea pig Cy3 (1:500; Jackson ImmunoResearch); and v) Rabbit anti-GFAP (1:1000; Dako) with Goat anti-rabbit Cy3 (1:500; Jackson ImmunoResearch).

In some instances, following secondary antibody application, DAPI (300 nM in PBS, Invitrogen) was applied to coverslips for 10 min at room temperature, followed by three, 5-min rinses in PBS. All coverslips were mounted in either ProLong Gold anti-fade reagent (Invitrogen) or Fluormount-G (Southern Biotech). No signal was detected for secondary antibodies when primary antibodies were omitted. We also tested the specificity of the secondary antibodies by applying a primary antibody, but replacing the appropriate with an inappropriate secondary antibody that was normally used to detect an excluded primary antibody. This control was devoid of signal as well.

Microscopy, Imaging, and Image Analysis

Images of cells in culture were acquired using an Olympus IX-71 inverted microscope through a 20x objective (NA = 0.4) with a Hamamatsu ORCA-ER CCD camera controlled by Olympus Microsuite software (v. 5.0). For NTS cultures triple-immunostained against TH, GAD67, and MAP2, neurons (MAP2⁺) were divided into three separate phenotypes: 1) TH⁺; 2) GAD67⁺; and 3) TH⁻/GAD67⁻, since TH⁺ and GAD67⁺ did not overlap. In separate experiments, sets of sister cultures were triple-immunostained with antibodies against three marker combinations (TH/GAD67/MAP2, TH/VGlu2/MAP2, and GAD67/VGlu2/MAP2) in order to determine the fraction of TH⁻/GAD67⁻ neurons that shows VGlu2 immunoreactivity. For this analysis, all MAP2⁺ neurons were counted on each coverslip (706.9 mm²) and the presence of immunoreactivity for the other markers was assessed for each MAP2⁺ neuron. Based on these findings, in a subset of experiments designed to examine dendritic morphology of the glutamatergic subpopulation of TH⁻/GAD67⁻ neurons, a combination of antibodies against TH, VGlu2, and MAP2 was used, and neurons with the VGlu2⁺/TH⁻ phenotype were selected for the analysis.

To assess dendritic morphology in all conditions and for each neurochemical phenotype, all NTS neurons present on a coverslip, or a maximum of 30 randomly chosen neurons, were imaged. For hippocampal cultures, 20 randomly chosen MAP2⁺ cells were analyzed per coverslip. Cells were excluded from the morphological analysis if: 1) vacuoles were apparent in the cell bodies, 2) all dendritic processes were shorter than 10 μ m, or 3) the dendritic arbors overlapped with dendrites of other neurons. In some instances, a process showed strong MAP2 immunoreactivity only in the proximal segment, with an abrupt loss of MAP2⁺ signal, followed by what morphologically appeared to be the axon. In these instances, the segment showing strong MAP2 immunoreactivity was included in the analysis if its length exceeded 10 μ m. In order to quantify dendritic arbors, the NeuronJ plug-in (Meijering et al., 2004) for ImageJ software (v. 1.44f, NIH) was used to trace dendrites and acquire three parameters for each neuron: number of primary dendrites, total dendritic length, and number of branch tips. For all neurons of a given phenotype, each dendrite parameter was averaged to acquire the mean per coverslip. A typical experiment consisted of at least three independent cultures prepared on different dates, with each culture containing 2–3 biological replicates (coverslips).

In order to assess the number of glial cells present in the NTS cultures, out of all images taken for MAP2 immunoreactivity in neurons, 15 images (the total area of 2.5 mm²) were selected such that they were evenly distributed across the coverslip. Contrast was enhanced in order to detect the background of glial cells and all glial cells were counted, followed by computing their average density (number per mm²). This method of analysis was verified with cultures triple-stained specifically for GFAP, MAP2, and DAPI. Two investigators (J.L.M. and A.L.B.) independently confirmed that the average density of glial cells obtained using the MAP2 background method did not significantly differ from the density of cells with DAPI-stained nuclei that were not MAP2⁺.

For purified astrocyte cultures used to generate ACM, five images (the total area of 0.8 mm²) were randomly sampled from a coverslip labeled for DAPI, and for GFAP and MAP2 immunoreactivities. To determine the purity of astrocytic cultures, DAPI and GFAP images were overlaid, and the number of cells with DAPI-positive nuclei that showed GFAP immunoreactivity was counted and expressed as a percentage of all cells with DAPI-positive nuclei. MAP2 immunoreactivity was used to confirm a lack of neurons. Cultures were used only if more than 95% of cells with DAPI-stained nuclei were GFAP^[+]. Typical cultures contained over 98% of astrocytes. In addition, the average density of astrocytes was computed and expressed per mm².

Statistical Analysis

One-way ANOVA followed by Tukey's post-hoc test was used to detect significant differences between groups when more than a single comparison was made. For studies involving BDNF treatment and glial depletion, a two-way ANOVA was used in order to determine whether a significant interaction occurred between the two factors. In all other instances, where a single comparison was made, an independent sample *t*-test was used. In all cases using ANOVA and *t*-test, data followed a normal distribution and had equal variances. When normality and/or variance equality was not met, Kruskal-Wallis test with pair-wise comparisons, or Mann Whitney U test, was performed. All data, unless otherwise specified, are expressed as mean $\pm$ SEM, and $p < 0.05$ was considered significant. PASW Statistics software (v. 18) was used for all statistical tests, except regression analysis, which was performed with GraphPad Prism (v. 5.02).

RESULTS

The *in vitro* model of the NTS

In order to test our specific hypotheses on the role of BDNF in the morphological maturation of visceral sensory relay neurons from the NTS, we developed a low-density dissociate culture model which faithfully reproduces neuronal phenotypes, including their relative proportions, that are found *in vivo*. The overwhelming majority of NTS neurons are second-order (McDougall et al., 2009), i.e. receive direct synaptic input from primary visceral afferents, which, based on our previous studies, constitute a significant endogenous source of BDNF (Martin et al., 2009). Moreover, electrophysiological studies have confirmed the predominantly second-order nature of two neurochemically distinct populations of NTS neurons: the catecholaminergic (Appleyard et al., 2007) and GABAergic (Bailey et al., 2008).

In the current study, both populations of second-order neurons were identified immunocytochemically by their specific markers, tyrosine hydroxylase (TH) and glutamic acid decarboxylase 67-kD isoform (GAD67), respectively. For each culture, antibodies against TH and GAD67 were combined with antibodies recognizing microtubule-associated protein 2 (MAP2), a general somatodendritic marker. Using this approach, we have found that, within the MAP2^[+] population, there was no overlap between TH^[+] and GAD67^[+] neurons (Fig. 1A), which is consistent with previous data obtained *in vivo* (Stornetta and Guyenet, 1999). Moreover, because TH and GAD67 immunoreactivities do not overlap, a distinct third population of neurons was possible to analyze: the MAP2^[+] population that lacks both TH and GAD67 immunoreactivities (TH^[-]/GAD67^[-]; Fig. 1A). In our cultures, among all MAP2^[+] cells, 29.86 $\pm$ 1.7%, 51.46 $\pm$ 2.0% and 18.6 $\pm$ 1.0% were TH^[+], GAD67^[+], and TH^[-]/GAD67^[-], respectively (Fig. 1B). Furthermore, we have determined that, for each of the three subpopulations, the average number of neurons per coverslip was not affected by our experimental conditions, including BDNF treatment and the density of glial cells (Table 1). Thus, the data presented below on dendritic morphogenesis of NTS

neurons are unlikely attributable to a phenotypic switch among neurons from neurochemically distinct populations.

When glia are abundant, BDNF selectively increases dendritic length and complexity of TH^[+] and TH^[-]/GAD67^[-], but not GAD67^[+], NTS neurons

We first tested the hypothesis that exogenous BDNF applied to cultured NTS neurons increases the outgrowth and complexity of their dendritic arbors. The experiments were designed to mimic the *in vivo* setting, where BDNF is released from primary afferents onto second-order neurons in the NTS. We grew mixed neuron-glia NTS cultures for a total of 7 days, and treated with either BDNF (200 ng/mL) or vehicle control during the final three days. We imaged MAP2^[+] neurons in each culture and assessed the number of TH^[+], GAD67^[+], and TH^[-]/GAD67^[-] cells within the MAP2^[+] population. To determine the effects of BDNF on dendritic growth and complexity, we quantified the number of primary dendrites, the total dendritic length, and the number of branch tips for each neuron.

In support of our hypothesis, TH^[+] neurons responded to BDNF by significantly increasing the average total dendritic length (Fig. 2A–B, Abundant Glia), the average number of branch tips (Fig. 2C, Abundant Glia), and the average number of primary dendrites per neuron (Control, 1.98 ± 0.08 , $n=180$ vs. BDNF-treated, 2.39 ± 0.08 , $n=191$, one-way ANOVA followed by Tukey's post-hoc comparison, $p=0.002$).

On the other hand, GAD67^[+] neurons showed no significant change in the average total dendritic length (Fig. 2D–E, Abundant Glia), the average number of branch tips (Fig. 2F, Abundant Glia), or the total number of primary dendrites in response to BDNF (Control, 2.28 ± 0.07 , $n=203$ vs. BDNF-treated, 2.48 ± 0.07 , $n=225$, one-way ANOVA followed by Tukey's post-hoc comparison, $p=0.2$).

Lastly, TH^[-]/GAD67^[-] neurons exhibited qualitatively the same response to BDNF as TH^[+] neurons, but its magnitude was much greater (the average total dendritic length, 43.5% increase compared to 25.1% in TH^[+] neurons; the average number of branch tips, 42.4% increase compared to 25.7% in TH^[+] neurons; the number of primary dendrites: Control, 2.33 ± 0.1 , $n=110$ vs. BDNF-treated, 3.16 ± 0.1 , $n=138$, one-way ANOVA followed by Tukey's post-hoc comparison, $p<0.001$; Fig. 2G–I, Abundant Glia).

These findings indicate that BDNF acts on subpopulations of NTS neurons to increase the length and complexity of dendritic arbors during the early postnatal period. Moreover, these data reveal differential sensitivities to BDNF-mediated dendritic growth among the three neurochemically-distinct populations.

Glia depletion changes the course of BDNF-mediated NTS dendritogenesis

An appreciation of neuron-glia interactions has grown substantially in recent years (Barres, 2008) and this holds true for the autonomic areas of the CNS (Gourine et al., 2010), including the NTS (Hermann et al., 2009). Throughout the first postnatal weeks, the number of astrocytes in the NTS rapidly increases (Pecchi et al., 2007). This suggests that glia may be involved in the postnatal maturation of autonomic pathways. To begin addressing this possibility, we next asked whether the effect of BDNF on NTS dendritic outgrowth is dependent on the presence of glia, by significantly depleting glial cells from the culture.

The numbers of glial cells in glia-depleted cultures were very significantly reduced (6- to 8-fold) compared to cultures with unmodified glial density (Kruskal-Wallis pairwise comparison, $p<0.01$; Table 1). Fascinatingly, minimizing the density of glial cells completely reversed the direction of the response of dendritic length and complexity to BDNF. Namely, under glia-depleted conditions, BDNF significantly decreased the average

total dendritic length in all three neuronal populations (Fig. 2A–B, D–E, G–H, Depleted Glia). The average number of branch tips was significantly decreased in TH⁺ and TH⁺/GAD67⁺ (Fig. 2C, I, Depleted Glia). In addition, the TH⁺/GAD67⁺ population responded with a decreased average number of primary dendrites per neuron (Control, 3.06 ± 0.1 , n=158 vs. BDNF-treated, 2.63 ± 0.1 , n=158, one-way ANOVA followed by Tukey's post-hoc comparison, p=0.016; Fig. 2H).

Thus, the presence or absence of abundant glia determines the direction and magnitude of the response to BDNF (two-way ANOVA: TH⁺ neurons, p<0.001, TH⁺/GAD67⁺ neurons, p<0.001). Moreover, GAD67⁺ neurons, whose dendritic morphology was not affected by BDNF in the presence of abundant glia (Fig. 2E, F, Abundant Glia), showed a significant decrease in the average total dendritic length upon glia depletion (Fig. 2E, Depleted Glia). However, the average number of branch tips (Fig. 2F, Depleted Glia) and the average number of primary dendrites per neuron (Control, 2.64 ± 0.07 , n=228 vs. BDNF-treated, 2.64 ± 0.07 , n=232, one-way ANOVA followed by Tukey's post-hoc comparison, p=1.0) remained unaffected in the GAD67⁺ population.

Overall, while BDNF changed the dendritic morphology of each neuronal population under glia-depleted conditions, the response of TH⁺/GAD67⁺ neurons was most pronounced and complete, with all three dendritic parameters being dramatically affected (Fig. 2G–I, Depleted Glia). Notably, the TH⁺/GAD67⁺ population was also most responsive to BDNF in the presence of abundant glia (Fig. 2G–I, Abundant Glia). For those reasons, we focused our subsequent analysis on this particular neuronal population.

A diffusible factor derived from astrocytes reduces dendritic outgrowth and complexity of NTS neurons

The experiments aimed at determining the influence of glia on NTS dendritogenesis mediated by BDNF revealed a novel and unexpected phenomenon. Namely, the glial cells themselves potentially reduce dendritic growth and complexity in all three populations of NTS neurons under control (no BDNF) conditions (Fig. 2, Control; Table 2, one-way ANOVA followed by Tukey's post-hoc comparison, all p-values <0.01). In support, for the TH⁺/GAD67⁺ population, which showed the most pronounced response to both BDNF and glial deprivation (Fig. 2G–I; Table 2), there was a strong negative linear relationship between the logarithm of the density of glial cells per culture and its corresponding mean total dendritic length per culture ($r^2=0.79$, p<0.001, n=15 cultures).

The wide range of glial cell densities from experiment to experiment was, in part, a result of a distinct culture treatment to obtain abundant glia (unmodified, mixed neuron-glia cultures) vs. glia-depleted conditions. The latter included application of the anti-mitotic agent cytosine β -D-arabino-furanoside (AraC, please see Methods for details). Thus, we next verified that the observed differences in neuronal morphologies between cultures with abundant and depleted glia can be attributed to differences in glial densities, and not to the AraC treatment and a resulting change in properties of glia and/or neurons. Here we examined the mean total dendritic length of TH⁺/GAD67⁺ neurons grown in AraC-treated cultures, and compared the results between the lowest (60–68 cells/mm²) and the highest (143–146 cells/mm²) glial densities. Despite the narrow, 2.3-fold, range of glial cell densities compared to the 6- to 8-fold differences between the abundant glia and glia-depleted conditions, the difference between the mean total dendritic length was significant for the BDNF-treated group (lowest glial density: $104.07 \pm 17.42 \mu\text{m}$, n=22; highest glial density: $187.14 \pm 17.04 \mu\text{m}$, n=43; p=0.003), and nearing the significance for the vehicle-treated control group (lowest glial density: $261.17 \pm 22.31 \mu\text{m}$, n=26; highest glial density: $215.71 \pm 14.69 \mu\text{m}$, n=49; p=0.083). These data indicate that the observed effects of glia on

NTS dendritic morphogenesis are attributable to the density of glia and not the AraC treatment.

We next determined the most likely candidate cell type that is mediating the inhibitory effect on NTS dendritic growth. Under both abundant (Fig. 3A) and depleted (Fig. 3B) glial cell conditions, the predominant cell type in our culture were astrocytes (abundant glia, $89.0 \pm 1.3\%$ vs. depleted glia, $87.3 \pm 1.5\%$, independent sample *t*-test, $p=0.5$, $n=3$ cultures). Even when astrocytes were significantly depleted from the culture, dendrites still tended to come into contact with astrocytes (Fig. 3B), which would support a contact-mediated mechanism of growth inhibition, with astrocytes providing a stop signal. However, arguing against this scenario is another observation that neuronal processes in astrocyte-abundant conditions grow over and past their first astrocytic contacts (Fig. 3A). Together, these observations led us to hypothesize that the reduced dendritic growth in NTS neurons is mediated by astrocytes through a diffusible factor.

In order to test this hypothesis, we treated NTS cultures that were depleted of glial cells with medium collected from purified astrocyte cultures from the NTS region (astrocyte-conditioned medium, ACM) or with non-conditioned medium as the control (Fig. 4A). If a diffusible factor from astrocytes is responsible for the reduced dendritic length and complexity of NTS neurons, ACM treatment in the relative absence of astrocytes (glia-depleted conditions) should mimic the effect of glia, i.e. lead to a decreased dendritic length and complexity compared to control neurons treated with the unconditioned medium.

Consistent with our hypothesis, ACM significantly reduced the total dendritic length (Fig. 4B), the number of branch tips (Fig. 4C), and the total number of primary dendrites per neuron (Control = 2.57 ± 0.73 , $n=254$ vs. NTS ACM-treated = 2.18 ± 0.68 , $n=218$ neurons, independent samples, *t*-test, $p<0.001$). These data indicate that the glia-dependent suppression of NTS dendritic morphogenesis is mediated by astrocyte-derived diffusible factor(s). Although still highly significant, the effect of ACM (Fig. 4) compared to the effect of abundant glia (Table 2) was noticeably smaller in magnitude. One likely explanation for this quantitative difference is instability of the diffusible factor which, once released from astrocytes, undergoes a gradual enzymatic and/or heat degradation if not bound to its biological target. We have further explored this possibility (please see the 'Heat-inactivation reverses the inhibitory effect of ACM on dendritic morphogenesis in NTS neurons' section below). Another potential explanation could be that the depletion of glia leads to functional changes in NTS neurons that include a decreased responsiveness to the astrocyte-derived diffusible factor(s).

Astrocyte-conditioned medium from NTS astrocytes, which inhibits NTS dendritogenesis, promotes dendritic outgrowth and complexity of hippocampal neurons

Our finding that ACM treatment reduces the dendritic length and complexity of NTS neurons is contrary to the canonical dendritic outgrowth-promoting response to ACM treatment exhibited by other CNS neurons (Banker, 1980). Therefore, we next asked whether the ACM derived from NTS astrocytes would retain its inhibitory effect on cultured hippocampal pyramidal neurons that have previously been shown to increase neurite growth in response to ACM derived from cortical astrocyte cultures (Banker, 1980). Compared to non-conditioned control medium, treatment with ACM derived from purified astrocyte cultures from the NTS region (Fig. 4D) dramatically increased the total dendritic length (Fig. 4E) and the number of branch tips (Fig. 4F). We have also examined the effects of treatment with ACM derived from purified *cortical* astrocyte cultures as in previous studies (positive control) and there were no discernable differences between the effects of cortical and NTS ACM (data not shown). Together, these data reveal that the effects of astrocyte-derived diffusible factor(s) are neuron type-specific.

Neurons whose dendritic morphology is most strongly affected by BDNF and glia are primarily glutamatergic

The central integration of autonomic reflexes that takes place in the NTS is based on the interactions between excitatory and inhibitory signals, derived primarily from glutamatergic and GABAergic neurons, respectively (Jin et al., 2004). While most of the GABAergic neurons in the NTS receive a direct synaptic input from primary visceral sensory neurons (Bailey et al., 2008), their *in vitro* counterparts (GAD67⁺ neurons) are either non-responsive or only weakly responsive to dendritogenic effects of BDNF, being in sharp contrast to the strong responsiveness of TH⁺/GAD67⁺ neurons (Fig. 2). Similarly, the effects of glia on TH⁺/GAD67⁺, compared to GAD67⁺ neurons are two-fold stronger (Table 2). We hypothesized that TH⁺/GAD67⁺ neurons, which not only constitute a significant fraction of all neurons in NTS culture but whose response to both BDNF and glia is most prominent, are glutamatergic.

A selective marker of NTS glutamatergic neurons is the vesicular glutamate transporter type 2 (VGlut2; Stornetta et al., 2002). In order to determine the fraction of TH⁺/GAD67⁺ neurons that contains VGlut2⁺, the following triple-immunocytochemistry protocols were applied to sister cultures: 1) TH, GAD67 and MAP2; 2) TH, VGlut2 and MAP2; and 3) GAD67, VGlut2 and MAP2. Since TH⁺ and GAD67⁺ neurons do not overlap, and VGlut2⁺ and GAD67⁺ neurons have a negligible overlap (1.6% ± 2.0%), the fraction of TH⁺/GAD67⁺ neurons that expresses VGlut2 (VGlut2⁺/TH⁺) is based on the percentage of VGlut2⁺/TH⁺ neurons (14.5%; Table 3) relative to the percentage of all TH⁺/GAD67⁺ (22.0%; Table 3). The results of this calculation show that a majority of TH⁺/GAD67⁺ neurons express VGlut2 (65.9% ± 6%; Fig. 5) and, therefore, are likely to be glutamatergic. Moreover, the fact that VGlut2 is expressed by nearly all TH⁺, but virtually none of GAD67⁺ neurons (Table 3; Fig. 5) is consistent with previous studies of NTS neurons *in vivo* (Stornetta et al., 2002), and provides an important validation of our *in vitro* NTS model. Significantly, these data indicate that the factors identified in our study as modulators of NTS dendritic morphology affect primarily excitatory neurons.

Heat-inactivation reverses the inhibitory effect of ACM on dendritic morphogenesis in NTS neurons

Having established that the majority of TH⁺/GAD67⁺ neurons, used in our earlier analysis of the effects of glia on dendritic morphology, express the glutamatergic marker VGlut2, we next aimed to verify that the VGlut2⁺ subpopulation of TH⁺/GAD67⁺ neurons is responsive to the inhibitory effects of ACM. In addition, the studies performed in the TH⁺/GAD67⁺ population suggested that the astrocyte-derived diffusible factor(s) that are mediating the inhibitory effect on NTS dendritic morphogenesis may be unstable and undergo degradation. To examine the heat-sensitivity of the ACM-derived factor(s), we exposed both ACM and control media to a mild heat-inactivation protocol (55°C, 30 min) that is commonly used to inactivate immune proteins that are found in serum, while leaving more stable proteins, such as growth factors, intact. Each day beginning on the 4th day *in vitro* (DIV), glia-depleted NTS cultures were treated with: ACM, control medium, heat-inactivated ACM, or heat-inactivated control medium. Cultures were fixed on the 7th DIV and immunostained for TH, VGlut2, and MAP2 (Fig. 6A). All VGlut2⁺/TH⁺ that were also MAP2⁺ were identified and imaged. Next, all dendritic parameters that have previously been described were measured using MAP2⁺.

Consistent with our earlier results, ACM significantly decreased the total dendritic length of VGlut2⁺/TH⁺ neurons compared to control medium (Fig. 6B). Notably, effects of heat-inactivated ACM were not different from unconditioned, heat-inactivated or non-inactivated control medium (Fig. 6B). Therefore, we conclude that an astrocyte-derived diffusible factor

underlying the inhibitory effect on the total dendritic length of NTS neurons is a heat-labile factor and, thus, likely a peptide or protein. The lack of heat stability argues against neurotrophins, such as BDNF, as likely candidate mediators since they are highly heat stable. In addition, our data indicate that VGlut2^[+]/TH^[-] neurons exhibit a similar response to treatment with ACM as TH^[-]/GAD67^[-] neurons, providing additional evidence for the role of diffusible astrocytic factor(s) in shaping the dendritic arbors of excitatory NTS neurons.

DISCUSSION

In the current study, we show for the first time that NTS neuron dendritogenesis is regulated by both BDNF and diffusible factor(s) released from astrocytes. Moreover, our study demonstrates a previously unknown interaction between BDNF and glia, with glia determining the course of dendritic morphogenesis mediated by BDNF. Consistent with previous studies in other parts of the brain, the magnitude of BDNF-mediated dendritic remodeling is dependent on neuronal phenotype. In addition, the inhibitory effect of astrocyte-conditioned medium on NTS dendritic growth and complexity reveals fundamental differences between this brain region and the hippocampus, whose neurons increase dendritic length and complexity under the same conditions (Banker, 1980).

Our finding that BDNF has differential effects on the dendritic growth of phenotypically distinct NTS neurons adds to the growing body of evidence that BDNF acts in a context-dependent manner. McAllister and colleagues were the first to show that BDNF could either increase or decrease dendritic growth depending on the cortical location of neuronal cell bodies (McAllister et al., 1995, McAllister et al., 1997). More recently, a targeted deletion of BDNF in post-mitotic neurons reveals that BDNF induces postnatal dendritic elaboration in a brain region-selective manner. Namely, BDNF is required for the dendritic growth of striatal neurons, while hippocampal neurons are almost entirely unaffected (Rauskolb et al., 2010). This finding was recapitulated in dissociate cultures from both striatum and hippocampus that were treated with BDNF. Based on these observations, the authors concluded that sensitivity to BDNF may be dependent on a neuron-intrinsic program (Rauskolb et al., 2010). Our present findings support this notion since the dendritic characteristics of neurons with three distinct phenotypes grown in the same culture are differentially affected by BDNF. However, we also find that the presence of glia may modulate this intrinsic program.

Our finding that dendrites of distinct neuronal populations are differentially modulated by BDNF treatment provides a framework for how an afferent-derived signal could result in shaping the neural circuitry of functionally distinct neuron types. It was previously shown in gustatory regions of the postnatal rat NTS that dendritic growth and complexity of neurons classified based on soma shapes were differentially modulated by reversibly blocking afferent activity through altering maternal dietary NaCl (King and Hill, 1993). It is well established that activity powerfully drives dendritogenesis and, in some cases, BDNF is the transducer of these activity-dependent changes. For example, blocking endogenous BDNF signaling abolishes activity-dependent dendritic remodeling in the *in vivo* retina (Landi et al., 2007). In fact, our previous data provide several lines of evidence that BDNF is poised to mediate afferent activity-dependent maturation in the NTS (Martin et al., 2009). Combined with our current findings that exogenous BDNF mediates phenotype-dependent responses of dendritic growth in NTS neurons, it is imperative to examine dendritogenesis in these distinct neuronal populations in the NTS *in vivo* following BDNF knockdown in early postnatal visceral afferents.

In addition to presynaptic activity, activity of the postsynaptic target neuron may determine its responsiveness to BDNF (Meyer-Franke et al., 1998). Electrical activity of neurons is a result of functional synapses that can also be formed between cultured neurons. However, our present study was designed to examine in detail dendritic morphology of individual neurons. Consequently, the neuronal density was set at a low level to prevent physical overlap of neurons, largely ruling out the possibility of synapse formation and activity as an additional factor shaping responses to BDNF in our model.

We find for the first time that, within the same neuron phenotype, the direction and magnitude of BDNF-mediated dendritic morphogenesis are determined by the number of glia. This finding is supported by previous studies that reveal complex neuron-glia interactions involving BDNF and/or TrkB (Djalali et al., 2005; Elmariah et al., 2005). For example, the postsynaptic clustering of GABA receptors in hippocampal neurons that is induced by a diffusible astrocyte-derived factor is prevented in neurons lacking BDNF or TrkB. This suggests that a diffusible astrocytic factor is required to form the inhibitory postsynaptic elements by modulating neuronal BDNF release and signaling through TrkB (Elmariah et al., 2005). In another study, BDNF added to glia-depleted cultures of serotonergic neurons from the raphe nucleus increases the number of primary dendrites, whereas simultaneously adding the astrocytic calcium-binding protein, S100 β , inhibits the BDNF-mediated dendritic outgrowth (Djalali et al., 2005). These data combined with our current findings highlight the need to understand neuron-glia interactions in mechanisms of neurotrophin action since both of these factors are established potent mediators of developmental events.

We have also found a previously unknown effect of diffusible factor(s) present in astrocyte-conditioned medium (ACM). Namely, NTS ACM reduces dendritic outgrowth of NTS neurons while promoting the outgrowth of hippocampal pyramidal neuron dendrites. Thus, the canonical dendritic growth-promoting response to astrocytic factors (Banker, 1980; McAllister, 2000) appears to be not universal but, instead, neuron type- and/or context-specific, with consequences for how we approach and interpret mechanisms of neuronal circuit formation in different parts of the brain. Time and again, diffusible, astrocyte-derived factors have been shown to promote dendrite growth (Powell et al., 1997; McAllister, 2000). Inhibitory actions due to diffusible factors have rarely been described (Lein et al., 2002) and, in most cases, it is unclear whether there is a true inhibitory factor or simply a lack of growth-promoting factor (Denis-Donini et al., 1984; Ballas et al., 2009). In our experiments, heat-inactivating the ACM completely abolished the inhibitory effect, strongly suggesting that the unknown factor is indeed inhibitory.

Our knowledge of diffusible factors released from astrocytes is growing, yet many remain to be identified. For example, the growth-promoting effect of astrocyte-derived factors on hippocampal neurons was first described more than three decades ago (Banker, 1980), yet the factor(s) remain unknown (Barres, 2008). Potential candidates include the soluble bone morphogenic protein (BMP) inhibitors, such as follistatin and noggin, that act by sequestering soluble, dendritic growth-promoting BMPs (Cho and Blitz, 1998). In sympathetic neurons, neuron-glia interactions influence the balance of BMPs and BMP inhibitors during the period of dendritic elaboration, with BMPs up-regulated and BMP inhibitors down-regulated, respectively (Lein et al., 2002). Another group of candidate molecules are astrocyte-derived factors that affect synaptogenesis, such as thrombospondins (Christopherson et al., 2005) and cholesterol (Mauch et al., 2001). In addition, a recent study has identified oleic acid as a neurotrophic factor derived from astrocytes of the periventricular zone that promotes axon formation in the striatum (Polo-Hernández et al., 2010). Critical questions that need to be addressed include determining whether the factor(s) that promote hippocampal dendritogenesis are the same or distinct from the factor(s) that

inhibit NTS dendritic outgrowth, as well as what are the receptors and/or downstream signaling pathways that may be involved.

Besides their importance to CNS development as a whole, our data bring up many intriguing implications for dendritogenesis in the NTS *in vivo*. Earlier studies have shown that, during the first week of postnatal development in rats, NTS dendrites extend from two opposite poles of the soma while orienting toward the solitary tract (Kalia et al., 1993), i.e. the source of afferent input. Thus, it is possible that the initial dendritic growth is guided by BDNF, which is vastly expressed in, and released from, the solitary tract afferents (Martin et al., 2009; Kline et al., 2010).

Following this initial directional growth, dendrites elaborate in all directions yet, intriguingly, are confined to only the ipsilateral NTS and the adjacent dorsal motor nucleus of vagus nerve (Kalia et al., 1993). Our current data, combined with the results of studies on spatial distribution of astrocytes in the brainstem, may provide an explanation for the restricted growth of NTS dendrites. Namely, at birth, while there are only few astrocytes within the NTS, its midline and dorsal borders are lined with densely packed astrocytes (Pecchi et al., 2007). Thus, our finding that astrocytes provide an inhibitory signal to the NTS dendritogenesis suggests that NTS dendrites *in vivo* may be prevented from extending into inappropriate regions by midline/dorsal astrocytes.

It has also been established that astrocytes, originally confined mostly to the NTS borders, proliferate and invade the NTS itself during the first weeks of postnatal life (Pecchi et al., 2007). Our data show that the presence of glia is conducive to BDNF-mediated stimulation of NTS dendritic growth and complexity. This suggests a role of the astrocytic proliferation within the NTS in promoting development of synaptic connections that is known to take place within the first postnatal weeks (Kalia et al., 1993; Segar, 1997; Vincent and Tell, 1997, 1999; Kawai and Senba, 2000; Lachamp et al., 2002).

Finally, our findings have profound implications for both normal and pathological neurodevelopment. During normal CNS development, glial cells are rapidly proliferating concurrently with dendritogenesis and synaptogenesis. Our current findings suggest that, if either the numbers and/or timing of glial cell proliferation deviate from their normal course, the formation of neural circuitry could be fundamentally altered. In the developing NTS, significant astrocytic gliosis has been documented in infants whose death was linked to SIDS (Biondo et al., 2004). Whether the gliosis is a cause or an effect is presently unknown, but it is believed that SIDS victims exhibit abnormal autonomic reflex responses during sleep. The fact that the NTS plays a crucial role in integration of autonomic reflexes (Andresen and Kunze, 1994), together with our present findings, suggests that gliosis contributes to abnormal development of autonomic circuitry that can result in SIDS. In light of the ubiquitous nature of glial proliferation and BDNF-mediated activity-dependent maturation, our findings have CNS-wide implications and highlight the need for a better understanding of interactions between neurotrophins and glia during development.

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LIST OF ABBREVIATIONS

ACM	astrocyte-conditioned medium
BDNF	brain-derived neurotrophic factor
DIV	days <i>in vitro</i>
GAD67	glutamic acid decarboxylase 67-kD isoform
GFAP	glial fibrillary acidic protein
MAP2	microtubule-associated protein 2
NTS	nucleus tractus solitarius
SIDS	sudden infant death syndrome
TH	tyrosine hydroxylase
VGlut2	vesicular glutamate transporter type 2
[+]	immunoreactive
[-]	non-immunoreactive

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Figure 1. The *Nucleus Tractus Solitarius* (NTS) dissociate culture model exhibits three neurochemically distinct and non-overlapping neuronal subpopulations

A, Micrographs of a single field of an NTS culture which was triple-immunostained for microtubule-associated protein type 2, a general somatodendritic marker (MAP2, green), tyrosine hydroxylase, a marker of catecholaminergic neurons (TH, purple), and glutamic acid decarboxylase 67-kD isoform, a marker of GABAergic neurons (GAD67, red). The merged image shows both TH- and GAD67-immunoreactive neurons, in addition to a neuron which does not express either marker (Merge, green). Scale bar, 50 μ m. **B**, Diagram showing a relative distribution of the three phenotypically distinct neuronal subpopulations within NTS cultures: TH-immunoreactive (TH⁺, purple), GAD67-immunoreactive (GAD67⁺, red), and TH-non-immunoreactive/GAD67-non-immunoreactive (TH⁻/GAD67⁻, green). Data are from seven independent cultures, the total of 3377 neurons (mean $\pm$ SEM: TH⁺, 29.86 $\pm$ 1.7%; GAD67⁺, 51.46 $\pm$ 2.0%; TH⁻/GAD67⁻, 18.6 $\pm$ 1.0%).

Figure 2. BDNF regulates dendritic outgrowth and complexity of NTS neurons in a phenotype- and glia-dependent manner

Representative TH⁺ (A), GAD67⁺ (D), and TH^{-/-}/GAD67^{-/-} (G) NTS neurons grown under glia-abundant (Abundant Glia) or glia-depleted (Depleted Glia) conditions and treated with vehicle (Control) or 200 ng/mL BDNF (BDNF). All images demonstrate MAP2 immunoreactivity, which was used for the morphometric analysis of the dendrites. Scale bars, 25 μ m. Mean total dendritic length (B,E,H) and mean number of branch tips (C,F,I) per neuron in TH⁺ (B,C), GAD67⁺ (E,F) and TH^{-/-}/GAD67^{-/-} (H,I) populations of NTS neurons grown in control (solid bars) or BDNF-treated (hatched bars) cultures with abundant (Abundant Glia) or depleted (Depleted Glia) glial cells; * p <0.05, ** p <0.01, *** p <0.001, one-way ANOVA with Tukey's post-hoc comparison. The average number of primary dendrites per neuron and the number of neurons used for the analysis are as follows: TH⁺ neurons: abundant glia control 1.98 ± 0.08 , $n=180$, abundant glia BDNF 2.39 ± 0.08 , $n=191$, depleted glia control 2.56 ± 0.07 , $n=183$, depleted glia BDNF 2.30 ± 0.08 , $n=196$; GAD67⁺ neurons: abundant glia control 2.28 ± 0.07 , $n=203$, abundant glia BDNF 2.48 ± 0.07 , $n=225$, depleted glia control 2.64 ± 0.07 , $n=228$, depleted glia BDNF 2.64 ± 0.07 , $n=232$; TH^{-/-}/GAD67^{-/-} neurons: abundant glia control 2.33 ± 0.1 , $n=110$, abundant glia BDNF 3.16 ± 0.1 , $n=138$, depleted glia control 3.06 ± 0.1 , $n=158$, depleted glia BDNF 2.63 ± 0.1 , $n=158$; from five independent cultures (data are presented as mean $\pm$ SEM).

Figure 3. Astrocytes are the predominant glial cell type in NTS cultures

Representative examples of NTS cultures with abundant (A) and depleted (B) glial cells, immunocytochemically stained for the astrocytic marker GFAP (red), the somatodendritic neuronal marker MAP2 (green), and DAPI (blue), which labels all cell nuclei. There are no discernable differences in the astrocyte appearance between the abundant and depleted glia conditions; scale bar, 50 μ m.

Figure 4. Astrocyte-conditioned medium (ACM) inhibits dendritic outgrowth of NTS neurons while increasing dendritic length and complexity of hippocampal neurons

A–C, NTS neurons were grown in glia-depleted cultures and treated for 3 days with unconditioned medium or ACM derived from purified NTS astrocyte cultures. Representative examples of TH^[-]/GAD67^[-] neurons treated with control medium (**A**, Control) or NTS ACM (**A**, NTS ACM); scale bars, 25 μm . Mean total dendritic length (**B**) and mean number of branch tips (**C**) per neuron; *** $p < 0.001$, independent samples *t*-test; the total number of primary dendrites per neuron: 2.57 ± 0.73 in control vs. 2.18 ± 0.68 in NTS ACM-treated cultures, $p < 0.001$; Control, $n = 254$; NTS ACM, $n = 218$ neurons, from four independent experiments (data are presented as mean $\pm$ SEM). **D–F**, Hippocampal neurons (MAP2^[+]) were grown in a dissociate culture and treated for 5 days with either control medium or ACM derived from purified NTS astrocyte cultures. Representative examples of neurons treated with control medium (**D**, Control) or NTS ACM (**D**, NTS ACM); scale bars, 50 μm . Median total dendritic length (**E**) and median number of branch tips (**F**) per neuron; *** $p < 0.001$, independent samples Mann Whitney U test; the total number of primary dendrites per neuron: 4.50 ± 1.18 in control vs. 5.00 ± 1.02 in NTS ACM-treated cultures, $p = 0.14$; Control, $n = 20$; NTS ACM, $n = 20$ neurons, from one set of sister cultures (data are presented as median $\pm$ 95% confidence interval).

Figure 5. The vesicular glutamate transporter type 2 (VGlut2) is expressed by a significant proportion of NTS neurons, including a large fraction of TH^[+]/GAD67^[+] neurons

A, Venn diagram representing the population breakdown of NTS neurons, based on triple-immunocytochemistry protocols applied to sister cultures. Nearly all ($97.8\% \pm 1.0\%$) TH^[+] neurons (purple) are also VGlut2^[+]. On the other hand, VGlut2^[+] is absent from virtually all GAD67^[+] neurons (red). Among TH^[+]/GAD67^[+] neurons, $65.9 \pm 6\%$ are characterized by immunoreactivity to VGlut2 (green). The remaining neurons ($13.9 \pm 3\%$ of all MAP2^[+] cells) do not express any of the three markers (grey, Unknown). Data are derived from four independent cultures and presented as mean $\pm$ SEM. **B**, Micrographs of a single field of an NTS culture triple-immunostained for TH, VGlut2, and MAP2 demonstrating three neurochemically distinct neurons: VGlut2^[+]/TH^[+] (large arrow), TH^[+]/VGlut2^[+] (small arrow), and TH^[+]/VGlut2^[+] (arrowhead); scale bar, 50 μ m.

Figure 6. Heat-inactivation of astrocyte-conditioned medium (ACM) derived from purified NTS astrocyte cultures reverses the inhibitory effect on total dendritic length in VGlut2^[+]/TH^[-] neurons

VGlut2^[+]/TH^[-] NTS neurons were treated for 3 days with intact control medium (Control), ACM, heat-inactivated ACM, and heat-inactivated control medium. **A**, Representative examples of VGlut2^[+]/TH^[-] NTS neurons under each condition; **B**, Mean total dendritic length per neuron; * $p < 0.05$, n.s., not significant, one-way ANOVA with Tukey's posthoc comparison; the difference between ACM and heat-inactivated ACM is not statistically significant ($p = 0.109$). Control $n = 130$, ACM $n = 129$, Heat-inactivated ACM $n = 131$, Heat-inactivated control $n = 82$ neurons, from three independent cultures (data are presented as mean $\pm$ SEM).

Table 1

The cell survival (**Average number of neurons per coverslip**) within individual neuronal phenotypes (**TH⁺**, **GAD67⁺**, **TH⁺/GAD67⁺**) and the relative contribution of individual phenotypes (**% of all MAP2⁺ neurons**) are not affected by either glial density (**Abundant Glia** vs. **Depleted Glia**) or treatment with **BDNF** (200 ng/mL; shaded grey areas). Independent samples Kruskal-Wallis test (from seven independent cultures): **TH⁺** neurons, $p=0.4$; **GAD67⁺** neurons, $p=0.8$; **TH⁺/GAD67⁺** neurons, $p=0.3$.

	Abundant Glia		Depleted Glia	
	Control	BDNF	Control	BDNF
Median number of glial cells per mm ²	778 ± 247	776 ± 218	124 ± 36	95 ± 39
TH ⁺	Average number of neurons per coverslip	% of all MAP2 ⁺ neurons	Average number of neurons per coverslip	% of all MAP2 ⁺ neurons
	39.3 ± 6.0	36.5% ± 4.3%	37.0 ± 4.8	25.8% ± 3.0%
GAD67 ⁺	37.0 ± 4.8	30.0% ± 2.5%	35.0 ± 2.6	29.9% ± 1.7%
	57.9 ± 12.1	48.2% ± 5.3%	70.3 ± 10.8	54.3% ± 3.8%
TH ⁺ /GAD67 ⁺	66.1 ± 9.9	51.3% ± 4.1%	59.1 ± 8.3	52.5% ± 2.1%
	17.1 ± 3.5	15.3% ± 1.7%	23.1 ± 2.9	18.6% ± 2.0%
	22.9 ± 2.5	18.8% ± 2.0%	26.0 ± 5.0	22.0% ± 2.0%

The difference in glial cell density between unmodified (Abundant Glia) and glia-depleted (Depleted Glia) cultures was significant (Kruskal-Wallis pairwise comparison, $p<0.01$). There were no differences in glial cell densities between vehicle-treated (Control) and BDNF-treated (BDNF) cultures (Kruskal-Wallis pairwise comparison: Abundant Glia, $p=1.0$; Depleted Glia, $p=0.8$). Data are presented as mean ± SEM.

Table 2

Depleting glia dramatically increases dendritic length and complexity of NTS neurons compared to conditions with abundant glia, regardless of neuron phenotype.

	Total dendritic length (μm)	Number of branch tips	Number of primary dendrites
TH^[+]	47.58 ± 6.28 ***	0.91 ± 0.16 ***	0.58 ± 0.11 ***
GAD67^[+]	64.48 ± 8.90 ***	0.94 ± 0.18 ***	0.36 ± 0.10 **
TH^[-]/GAD67^[-]	111.69 ± 13.24 ***	1.80 ± 0.30 ***	0.74 ± 0.16 ***

Results presented are mean differences (± SEM) in untreated control between glia-depleted and abundant glia (Fig. 2) cultures;

**
p<0.01,

p<0.001, one-way ANOVA with Tukey's post-hoc comparison; TH^[+] neurons, depleted glia n=183, abundant glia n=180; GAD67^[+] neurons, depleted glia n=228, abundant glia n=203; TH^[-]/GAD67^[-] neurons, depleted glia n=158, abundant glia n=110, from five independent cultures.

Table 3

The relative contribution of individual phenotypes (% of all MAP2^[+] neurons) in sister cultures triple-immunostained with antibodies against selected immunochemical markers, applied in pairs (TH+GAD67; TH+VGlut2; GAD67+VGlut2), each combined with anti-MAP2. The presence of immunoreactivity for each marker was assessed for every neuron in culture (surface area of 706.9 mm²).

Immunochemical marker combination	Specific markers (all MAP2 ^[+])	% of all MAP2 ^[+] neurons	Number of cultures [MAP2 ^[+] neurons per culture]
TH + GAD67 + MAP2	TH ^[+] /GAD67 ^[+]	0%	n=5 [95.6 ± 18.6]
	TH ^[+] /GAD67 ^[-]	36.1 ± 16%	
	TH ^[-] /GAD67 ^[+]	41.9 ± 11%	
	TH ^[-] /GAD67 ^[-]	22.0 ± 6%	
TH + VGlut2 + MAP2	TH ^[+] /VGlut2 ^[+]	40.8 ± 19%	n=6 [100.7 ± 26.1]
	TH ^[+] /VGlut2 ^[-]	0.9 ± 0.7%	
	TH ^[-] /VGlut2 ^[+]	14.5 ± 6%	
	TH ^[-] /VGlut2 ^[-]	43.8 ± 11%	
GAD67 + VGlut2 + MAP2	GAD67 ^[+] /VGlut2 ^[+]	1.6 ± 2%	n=6 [100.2 ± 15.9]
	GAD67 ^[+] /VGlut2 ^[-]	38.8 ± 17%	
	GAD67 ^[-] /VGlut2 ^[+]	45.7 ± 15%	
	GAD67 ^[-] /VGlut2 ^[-]	13.9 ± 3%	

Results presented are mean numbers (± SEM) in untreated, glia abundant control cultures.